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REPORT

Contract : Number

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IDENTIFICATION OF SOURCES, PATHWAYS INTO A WELL AND PREVENTION FROM THE RISK OF HAVING PATHOGENS ENTERING ABSTRACTION WELLS

Project acronym: WellMa1

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for
Kompetenzzentrum Wasser Berlin gGmbH

Preparation of this report was financed in part through funds provided by BWB and Veolia

Berlin, Germany
2009

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Title

Identification of sources, pathways into a well and prevention from the risk of having pathogens entering abstraction wells

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Deliverable number

D 4.1

Abstract

This report attempts to give a survey from literature on the microorganisms involved, on the factors and mechanisms potentially relevant for the susceptibility of drinking water wells to health related microbial contamination.

The habitat groundwater accommodates a rich diversity of microorganisms, which has only begun to be identified since the development of molecular detection methods in addition to the conservative cultivation techniques.

Characteristics of the subsurface are darkness, low spaces, low nutrient and low oxygen content. Indigenous microorganisms have adapted to these oligotrophic conditions and are able to proliferate in this environment permanently. Other incoming microorganisms generally cannot reproduce under these conditions, but have developed strategies to survive. They can grow only, when the parameters turn favourable.

Pathogenic microorganisms comprise bacteria, viruses, and protozoa, which can also survive a certain time in groundwater. Most microorganisms in the subsurface are attached to surfaces and survive best within biofilm populations.

Pathogenic microorganisms originate from human or animal faeces. These organisms are not easily detected. The methods are very time and labour consuming. Therefore, other microorganisms regularly present in the faeces are used for detection. Their presence indicates the possibility of a contamination with pathogens. As indicator microorganisms mostly coliform bacteria, *E. coli*, enterococci and clostridia are used.

Contamination with pathogens is reported to derive essentially from communal sources: defects in wastewater treatment plants, sewage tanks, pipes, and waste deposits; from agricultural sources: animal wastes, liquid manure, and grazing; and from point sources like faeces from animals, birds, and humans. Entrance into the subsurface occurs via rainwater and surface waters, as well as by direct contamination of wells.

The transport of the microorganisms into the subsurface is influenced by the geologic conditions of a specific site: soil and rock type, presence of fissures, heterogeneity. In sand, microbial movement is less far than e.g. in Karst regions, thus the susceptibility to contamination of groundwater and wells is lower. Pore sizes are crucial for sedimentation and filter efficiency of the soil. Also important is the extent of the unsaturated zone, the flow velocity of the groundwater, the geochemistry and mineralogy of the site.

Wells receive their water from the groundwater reservoir of the surrounding soil. The quality of the well water is therefore essentially dependent on the properties of the groundwater and all the factors influencing the groundwater may also be relevant for the well water. The wells represent, in addition, a separate complex system with specific conditions and influencing parameters. This specific habitat involves additional variable adsorption surfaces, more space, higher flow velocity of the water, a mixing of waters from different groundwater layers and thus a different chemical composition. Contamination may also arise from microbial introduction at the open wellhead.

Two main processes have been identified which are essentially responsible for the elimination of pathogens during their pathway from top of the soil to the extraction well: inactivation of the microorganisms and their adsorption to the soil particles in the subsurface.

Both processes are influenced by a variety of factors and conditions present at a given site. To mention are here properties of

- the soil: consistence and texture of surfaces, electric charge, hydrophobicity, degree of moisture, coating with organic material

- the groundwater: temperature, pH, presence of cations and ionic strength, presence of organic substances, dissolved oxygen content, activity of indigenous microorganisms
- the microorganisms: forming of flagella, fimbria, hydrophobicity of the cell surface, forming of extracellular polymeric substances, forming of cysts and spores as survival strategies.

In addition to the description of the microbial diversity in the subsurface, the sources of pollution and the factors controlling the microbial pathways into groundwater and wells, main methods for the detection of a variety of contaminating microorganisms are given at the end of the report.

Kurzbeschreibung

Der vorliegende Bericht enthält die Ergebnisse einer Literaturrecherche über eine Gefährdung von Trinkwasserbrunnen durch gesundheitsrelevante mikrobielle Verunreinigung, mit dem Schwerpunkt auf den beteiligten Mikroorganismen sowie den beeinflussenden Faktoren und Mechanismen.

Innovative Forschung in diesem Bereich wurde erst durch die Entwicklung molekularer Nachweismethoden in Erweiterung zu den herkömmlichen Kultivierungsansätzen ermöglicht. Dies hat dazu geführt, dass in den letzten Jahren für den Lebensraum Grundwasser eine Vielzahl an Organismen nachgewiesen werden konnte, die vorher nicht bekannt waren.

Grundwasser als Habitat zeichnet sich durch Dunkelheit, räumliche Enge, geringen Nährstoffgehalt und niedrige Sauerstoffkonzentrationen aus. Indigene Mikroorganismen haben sich an diese oligotrophen Bedingungen angepasst und können sich dauerhaft ansiedeln. Eingetragene Mikroorganismen hingegen können sich i. d. R. unter diesen Bedingungen nicht vermehren, haben jedoch spezielle Überlebensstrategien entwickelt und vermehren sich nur dann, wenn die Parameter für sie günstig sind.

Zu den pathogenen Mikroorganismen zählen Bakterien, Viren und Protozoen, die ebenfalls eine gewisse Zeitspanne im Grundwasser überleben können. Sie besiedeln meist die inneren Oberflächen eines Grundwasserleiters. Dabei ist ihre Überlebensrate höher, wenn sie Teil eines Biofilms sind. Quelle der pathogenen Mikroorganismen sind Ausscheidungen von Mensch und Tier. Da die Bestimmungsmethoden zeit- und arbeitsintensiv sind, benutzt man andere Mikroorganismen, die regelmäßig im Stuhl vorkommen, als Indikatororganismen. Als solche Indikatormikroorganismen werden meist coliforme Bakterien, *E. coli*, Enterokokken und Clostridien verwendet.

Die Verunreinigung mit pathogenen Keimen lässt sich auf Einträge aus kommunalen Quellen zurückführen, wie beispielsweise Defekte in Abwasseranlagen, Tanks, Leitungen und Mülldeponien; aus landwirtschaftlicher Nutzung, wie beispielsweise Tierabfälle, Ausbringung von Gülle, Beweidung; und aus Punktquellen, die aus Exkrementen von Menschen oder Tieren herrühren. Der Eintrag in den Untergrund erfolgt zusammen mit dem Regen- und Oberflächenwasser oder durch direkte Kontamination am offenen Brunnen.

Der Transport der Mikroorganismen in den Untergrund wird durch die geologischen Bedingungen des jeweiligen Standorts beeinflusst: Die Beschaffenheit des Bodens und die Gesteinsart, das Vorhandensein von Spalten und Heterogenität. In sandigem Untergrund ist die Wanderung der Mikroorganismen geringer als z. B. in Karstregionen. Daher ist hier auch die Anfälligkeit von Grundwasser und Brunnen gegenüber Verunreinigungen geringer. Entscheidend für die Filterwirksamkeit des Untergrunds ist die Porengröße. Wichtig ist darüber hinaus die Schichtdicke der ungesättigten Zone, die Fließgeschwindigkeit des Grundwassers, die Geochemie und Mineralogie des Standorts.

Brunnen fassen Wasser aus dem Grundwasser ihres Umfeldes. Die Qualität des Brunnenwassers ist damit wesentlich geprägt von den Eigenschaften des Grundwassers und alle Faktoren, die das Grundwasser beeinflussen, wirken sich auch auf das Brunnenwasser aus. Zusätzlich stellen Brunnen einen eigenen Lebensraum mit komplexen Bedingungen und Einflussparametern. Dieses spezifische Habitat enthält zusätzliche Adsorptionsoberflächen, größere Räume, eine schnellere Fließgeschwindigkeit des Wassers, eine Mischung von Wässern aus unterschiedlichen Grundwasserschichten und damit auch eine unterschiedliche chemische Zusammensetzung des Wassers. Eine Verunreinigung kann zusätzlich auch aus direktem Eintrag am offenen Brunnen erfolgen.

Für die Elimination von pathogenen Keimen während ihrer Wanderung von der Oberfläche zum Trinkwasserbrunnen sind zwei grundlegende Prozesse verantwortlich: Inaktivierung der Mikroorganismen und ihre Adsorption an das Gestein des Grundwasserleiters. Beide Prozesse werden von einer Vielzahl von Faktoren und Bedingungen eines Standorts beeinflusst, wie z. B. den Eigenschaften

- des Bodens: Struktur und Beschaffenheit der Oberflächen, elektrische Ladung, Hydrophobizität, Feuchtigkeitsgehalt, Belag mit organischen Substanzen
- des Grundwassers: Temperatur, pH-Wert, Vorhandensein von Kationen und Ionenstärke, Anwesenheit von organischen Substanzen, Konzentration von gelöstem Sauerstoff, Aktivität von indigenen Mikroorganismen
- der Mikroorganismen: Oberflächenstrukturen mit Geißeln und Fimbrien, Hydrophobozität der Zelloberfläche, Bildung von extrazellulären polymeren Substanzen, Bildung von Zysten und Sporen als Überlebensstrategie.

Neben der Beschreibung der mikrobiellen Diversität im Untergrund, der Quellen der Verunreinigung und der Faktoren, die die Wanderung der Mikroorganismen in das Grundwasser und die Brunnen bestimmen, enthält der Bericht auch die wichtigsten Detektionsmethoden für eine Reihe von kontaminierenden Mikroorganismen.

Rèsumè

Ce rapport rassemble des données issues de la littérature autour de la problématique sanitaire de vulnérabilité des puits d'eau potable face à la contamination microbiologique. Il passe en revue les microorganismes impliqués dans cette contamination, ainsi que les potentiels facteurs et mécanismes associés.

Le développement des méthodes de détection moléculaires a permis, en complément des techniques de culture courantes, d'identifier la grande diversité des microorganismes qu'hébergent les nappes phréatiques. Les paramètres caractéristiques du sous-sol sont l'obscurité, des espaces très restreints, et de faibles concentrations en éléments nutritifs et en oxygène. Les microorganismes indigènes se sont adaptés à ces conditions oligotrophiques et sont capables de proliférer de manière durable dans cet environnement. Les autres microorganismes ne peuvent généralement pas se reproduire dans de telles conditions, mais ont néanmoins développé certaines stratégies de survie. Leur croissance ne devient possible que lorsque les paramètres favorables sont réunis. On entend par microorganismes pathogènes les bactéries, les virus, et les protozoaires, ces derniers étant capables de survivre pendant un certain temps dans les eaux souterraines. La majeure partie des microorganismes évoluant dans ce milieu demeurent fixés à des surfaces par adsorption, et les biofilms leur fournissent les meilleures conditions de survie.

Les microorganismes pathogènes proviennent des excrétions humaines et animales. N'étant pas faciles à détecter, et les méthodes associées étant particulièrement lentes et laborieuses, on utilise d'autres microorganismes régulièrement présents dans les selles, et dont la détection nous indique si une contamination par des germes pathogènes aurait pu avoir lieu. Les microorganismes indicateurs les plus utilisés sont les bactéries coliformes, E. coli, enterococci et clostridia. L'origine de cette contamination par des agents pathogènes est essentiellement communale (défauts dans les installations liées au traitement des eaux usées, réservoirs, conduites, décharges), agricole (déjections animales, épandage de lisier, pâturage), mais aussi d'origine plus ponctuelle lorsqu'elle concerne les excréments d'animaux, d'oiseaux, et d'hommes. Ces agents pathogènes pénètrent dans le sous-sol via les eaux de pluie et de surface, ou plus directement par contamination directe au niveau des puits ouverts.

Le transport des microorganismes dans le sous-sol dépend du contexte géologique de la région considérée: la qualité du sol et le type de roche, la présence de fissures et d'hétérogénéités. Dans le sable, le mouvement des microorganismes s'effectue plus lentement que dans les zones karstiques, ce qui diminue la vulnérabilité des eaux souterraines et des puits à toute contamination microbienne. La taille des pores est un facteur déterminant pour la l'efficacité de filtration et de sédimentation du sous-sol. L'étendue de la couche non saturée est aussi un élément important, de même que la vitesse d'écoulement de la nappe, la géochimie, et la minéralogie du site.

L'eau extraite par les puits provient du réservoir aquifère environnant, par conséquent sa qualité dépend principalement des propriétés de ce réservoir, et tous les facteurs l'influençant sont donc eux aussi à prendre en compte. En outre, un puits est un système complexe qui impose ses propres conditions et paramètres d'influence. Cet environnement spécifique permet la création de différentes surfaces d'adsorption et de nouveaux espaces, intensifie les vitesses d'écoulement et provoque un mélange inattendu d'eaux d'horizons stratigraphiques différents, modifiant alors leur composition chimique. La contamination peut aussi avoir lieu par introduction microbiologique directe en tête de puits.

L'inactivation des microorganismes et leur adsorption aux particules du-sous-sol ont été identifiées comme étant essentiellement les deux phénomènes responsables de

l'élimination des agents pathogènes lors de leur cheminement entre la surface du sol jusqu'aux les puits d'extraction. Sur un site donné, ceux-ci peuvent être influencés par une multitude de facteurs et de conditions. Ci-dessous sont mentionnées des propriétés :

- du sol: structure et qualité des surfaces, charge électrique, hydrophobie, taux d'humidité, recouvrement par des éléments organiques
- de la nappe phréatique: température, pH, présence de cations et force ionique, présence de substances organiques, teneur en oxygène dissout, activité des microorganismes indigènes
- des microorganismes: structures à flagelles et fimbriae, hydrophobie de la surface externe de la cellule, fabrication de substances polymères extracellulaires, création de kystes et de spores en tant que stratégie de survie.

Outre la description de la diversité microbienne du sous-sol, des sources de contamination et des facteurs qui commandent le parcours des microorganismes dans les nappes phréatiques et les puits d'extraction, le rapport s'intéresse aussi à désigner les méthodes importantes de détection de toute une liste de microorganismes concernés.

Acknowledgements

The Authors are grateful to all investigators for their research on this field. We are grateful for valuable discussions and informations from J. Lopez-Pila, P. van der Wielen, A. Bartetzko, and U. Szewzyk.

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Chapter 1
Introduction

The WellMa project, aiming at the optimization of operation and maintenance of drinking water abstraction wells, contains quantitative aspects as well as the assessment and possible improvement of the quality of the abstracted raw water. From together four work packages, WP 4 addressed the question if there is an impact from well operation & maintenance on the potential for *microbial contamination*.

This issue was pointed out by the Berliner Wasserbetriebe (BWB) after single events of overstepping limit values of the German drinking water ordinance due to Enterococci outbreaks. The BWB observed a possible coherence to construction work or pump replacement, but the detailed causes were not well understood up to now. To prevent such contaminations, the source of contaminants and their pathways into a well had to be investigated.

From a bibliographic study on existing cases of microbial well contamination, published cases should be compared to the situation in Berlin to analyse the possible origins of microbial contamination. Therefore, the following issues had to be investigated:

- Identification of relevant bacteria
- Evaluation of sources
- Assessment of pathways into a well
- Parameters from well design or well operation that increase the risk of contamination
- Techniques and analytical methods that can be used to determine the sources and pathways

Worldwide publications could be found dealing with microorganisms within the groundwater. Hence, it is by no means an unfavourable environment, quite the contrary, many bacteria have adapted to the special conditions present underground. The following report summarizes the findings of the literature review.

Chapter 2
Bacteria in groundwater and wells

2.1 Microbial diversity in aquifers

Aquifers are heterogeneous regarding their geographical and geological situation as well as regarding their saturated/unsaturated phases, the groundwater flow, and the interstitial water present. They provide heterogeneous conditions for colonization and reproduction of microorganisms. Bacterial populations in these habitats usually are quite heterogeneous as well (Brockman & Murray, 1997; Goldscheider et al., 2006).

2.1.1 Pristine environment

Even in unpolluted groundwater environments, some microorganisms are always present. Their persistence relies on the more or less constant supply of nutrient conditions and other physical, chemical, and community structures, which are typical for that ecosystem. General characteristics of groundwater habitats are small void spaces, darkness, low nutrient supply (either as organic detritus or dissolved substances), none or low oxygen concentrations, and low temperatures. Microorganisms persisting under such oligotrophic conditions have developed specific adaptation strategies. The absence of light requires the use of chemical sources for their energy need, resulting in chemotrophic metabolism where energy is derived from redox reactions, based either on inorganic substances (e. g. NH_3, H_2S, S, Fe^{2+}) or on organic materials (proteins, aminoacids) available in this environment.

Cells are usually small with low metabolic activity accompanied by low growth rates. As very often only limited oxygen is available, the microorganisms are forced to use anaerobic metabolic pathways with lower energy yield. They survive nutrient depletion or other temporary changes of water chemistry by switching metabolic pathways or reducing metabolic activities to levels of dormant states. Besides these physiologic features, also morphological changes may occur, like the increased production of extracellular polymeric substances. The presence of these structures might be advantageous for retaining nutrients and enhance attachment to solid surfaces resulting in the formation of biofilms, which offer protection against adverse chemical conditions and predation (Gounot, 1994). An indication of the adaptation to nutrient limitations is represented by accumulation of diverse storage inclusions in the cells (e.g. poly-ß-hydroxybutyrate, polyphosphates or polysaccharides) frequently encountered in indigenous groundwater microorganisms (Ghiorse & Balkwill, 1983).

The subject of microbial communities in pristine groundwater had long been neglected in research. It was first addressed by Wolters & Schwartz (1956) who identified gram-negative and gram-positive bacteria and different species of *Micrococcus*, *Achromobacter*, *Flavobacteria*, *Cytophaga*, and *Nocardia* based on cultivation and morphology. Thereafter members of different genera like *Microcylus*, *Prosthecomicrobium*, *Caulobacter*, *Hyphomicrobium*, *Planctomycetes*, *Gallionella*, *Agrobacterium*, *Clostridium*, and *Nocardia* have been identified based on cell morphology without cultivation (Hirsch & Rades-Rohkohl, 1983).

Microbial community structure in deep anaerobic aquifers has been analysed by taxon specific probes resulting in the detection of mostly Eubacteria including gram-positive bacteria, Deltaproteobacteria, *Desulfobacter*, and *Desulfovibrio* and a low number of Archaea (Fry et al., 1997).

Murakami et al. (2003) assayed groundwater at various depths and showed that different community structures could be detected at these sites by cultivation**,** including sulphate-reducing and sulphur-oxidizing bacteria, nitrate-reducing and denitrifying bacteria.

A clear correlation to depth could not be established, confirming the heterogeneous availability of substrates, electron donors and acceptors at the different sampling points. A cultivation based investigation of the saturated zones of two aquifers showed that the variety of aerobic colony types decreased with increasing saturation as determined by cultivation on nutrient-rich media. However, the colony counts were usually lower on high nutrient media for samples from saturated zones (Balkwill & Ghiorse, 1985). Different physiological microbial types have been detected in groundwater of different geothermic sites, which reveal a predominance of anaerobes over aerobes and thermophiles over mesophiles (Daumas, 1986).

In analyses using direct light microscopy, the most abundant bacteria were found to be gram-positive coccoid *Arthrobacter*-like species whereas the cultivation and isolation of bacteria from the same habitat resulted in the detection of a higher proportion of gram-negatives (Balkwill & Ghiorse, 1985). Gram-positive bacteria also predominated in indigenous groundwater communities detected by molecular methods (Wilson et al., 1983). Ultee et al. (2004) identified cultivable and uncultivable microorganisms in groundwater and found that the cultivable components belonged to Alpha-, Beta-, and Gammaproteobacteria, Flavobacteria, and Actinobacteria, most of the uncultured belonged to Betaproteobacteria.

Stetzenbach et al. (1986) examined bacteria in the water of two continuously pumping wells and were able to isolate predominantly *Acinetobacter* species, but also *Flavobacteria*, *Moraxella*, and *Pseudomonas/Alcaligenes* species and phylogenetically unidentified gram-positive rods and cocci. *Acinetobacter* were detected in a number of groundwater supplies on selective media by Bifulco et al. (1989). The microbial consortia in wells are also necessarily adapted to low nutrient concentrations and *Aeromonas*, *Flavobacteria*, and *Pseudomonas* species have been shown to multiply in oligotrophic waters (van der Kooij & Hijnen, 1981;van der Kooij et al., 1980).

Surveys on the microbial taxonomic and morphologic diversity in groundwater have been presented by Hirsch et al. (1992) and Preuss & Schminke (2004). The taxonomy of a number of bacteria detected in groundwater is presented in Table 1.

Different microbial occurrence in water and sediment. Morphological and physiological properties of isolates were determined from different depths of saturated groundwater by Koelbel-Boelke et al. (1988). Gram-negative isolates were more frequently found in all water samples whereas the gram-positive bacteria were mostly detected in sediment. The number of bacteria detected in groundwater was consistently much lower than in sediment samples (Preuss & Nehrkorn, 1988). Microbial abundance, measured by viable cell counting, declined with increasing depth (Ghiorse & Wilson, 1988). A quantitative estimate of microbial diversity has been attempted by exposing sterile sediments to a groundwater flow. The bacterial colonization was analysed and a variety of morphotypes were detected in the sediment fraction but less in the fluid fraction (Hirsch & Rades-Rohkohl, 1990) showing that many bacteria were attached to soil particles.

Bacteria isolated from groundwater of an aquifer associated with river bank filtration were identified as Alpha-, Beta-, Gamma-, and Deltaproteobacteria, Actinobacteria and Firmicutes as well as low numbers of manganese-reducing *Aeromonas* species, in contrast to sediment samples with only Gammaproteobacteria isolates.

Analyses of the groundwater interface showed that it harboured a higher number of microorganisms as compared to the vadose zone (Madsen & Ghiorse, 1993). The *in vitro* metabolic activities were also different in each community.

Table 1: Phylogenetic affiliation of bacteria isolated from groundwater

Class	Genus	Class	Genus
Acidobacteria	*Geothrix*	Alphaproteobacteria	*Agrobacterium*
Actinobacteria	*Arthrobacter*		*Azospirillum*
	Aureobacterium		*Blastobacter*
	Clavibacter		*Caulobacter*
	Corynebacterium		*Erythromicrobium*
	Frankia		*Hyphomicrobium*
	Gordonia		*Methylobacterium*
	Microcella		*Microcyclus*
			(Ancylobacter)
	Micrococcus		*Paracoccus*
	Mycobacterium		*Prosthecomicrobium*
	Nocardia		*Rhodobacter*
	Rhodococcus		*Sphingomonas*
	Rothia	Betaproteobacteria	*Achromobacter*
	Streptomyces		*Alkaligenes*
	Terrabacter		*Chromobacterium*
Flavobacteria	*Flavobacterium*		*Comamonas*
Sphingobacteria	*Cytophaga*		*Gallionella*
	Flectobacillus		*Leptothrix*
	Flexibacter		*Rhodocyclus*
Bacilli	*Bacillus*		*Telluria*
	Staphylococcus		*Thiobacillus*
Clostridia	*Clostridium*		*Variovorax*
Planctomycetes	*Planctomyces*		*Zoogloea*
		Deltaproteobacteria	*Desulfovibrio*
			Desulfuromonas
			Geobacter
			Pelobacter
		Gammaproteobacteria	*Acinetobacter*
			Aeromonas
			Citrobacter
			Enterobacter
			Moraxella
			Pseudomonas
			Shewanella
			Vibrio
			Xanthomonas

Adapted from Preuss & Schminke (2004).

⇨ The variety of micro-organisms attached to the aquifer matrix is higher than in the fluid phase of pristine groundwater.

Availability of nutrients, oxygen, and energy sources. Investigations of different pristine aquifers have underlined the importance of the available nutrients for the occurrence of microorganisms. The availability of different nutrients can be very heterogeneous and thus the composition of the microbial communities, e.g. where ferrous iron is present, iron-oxidizing bacteria may proliferate, in sulfide-rich waters Thiobacteria may frequently be found, the presence of soluble nitrogen may favour growth of nitrifying bacteria. Often the subsurface contains sufficient amounts of oxygen to allow the growth of facultative anaerobic and microaerophilic bacteria.

Pristine groundwater usually provides low concentrations of phosphate and less than 1mg/L dissolved organic carbon (Ghiorse & Wilson, 1988). Easily degradable organic matter is usually consumed by surface microorganisms, what is left are humic substances and other complex compounds, which are degraded by heterotrophic subsurface bacteria. Autotrophic bacteria use the dissolved carbon dioxide present in groundwater (Gounot, 1994). Boyd et al. (2007) exposed different geological substrates to groundwater in a pristine aquifer and analysed the developing biofilm. The community was found to be dominated by Proteobacteria, but the exposure of different substrates resulted in the growth of bacteria belonging to different subdivisions within this genus. Preferential colonization of bacteria (in groundwater) was observed on Feldspars which carried inclusions of phosphate minerals; enhanced bacterial growth was detected in microcosms containing silicates with iron oxide inclusions, showing that these substrates can be released and used by bacteria (Rogers & Bennett, 2004; Rogers, 2002; Rogers et al., 1998). Predominantly sulphur-oxidizing bacteria were detected in a subsurface sulfidic karst habitat (Engel, 2007).

⇨ Geochemistry and hydrochemistry determine the microbiological community in pristine groundwater.

Size adaptation. In the vegetative life cycle, bacterial cells have diameters of 1-10µm. When exposed to starving conditions they shrink to diameters of 0.1-0.5µm and become unattachable. Miyoshi et al. (2005) specifically selected small size microorganisms from deep groundwater by passing the water through 0.2µm pore size filters and analysed the microbial community in the filtrate by 16S rRNA cloning. A majority was identified to belong to the class Betaproteobacteria. It was shown that a substantial portion of the bacteria persisting in this oligotrophic habitat have adapted to the conditions by reduction of their cell size and by resting in a viable but not cultivable state (VBNC). In this state, the metabolism of the bacteria is heavily reduced and they cannot be detected by cultivation. Filterable bacteria through 0.45µm pore size in contrast were most frequently identified as Pseudomonads (Shirey & Bissonnette, 1991).

⇨ Micro-organisms in groundwater may rest in a viable but not cultivable state (VBNC), making them difficult to identify.

2.1.2 Chemically contaminated sites

Microbial diversity has been described in habitats where specific bacterial consortia have settled in chemically contaminated environments (Cavalca et al., 2004; Haack et al., 2004a; Hohnstock-Ashe et al., 2001; Kaestner et al., 2006; Madsen et al., 1991; Nakagawa et al., 2002; Watanabe et al., 2002) exploiting the specific nutrients or chemical conditions provided. Plate counts from polluted aquifer regions with high levels of usable carbon sources are mostly higher than those from uncontaminated sites are (Gounot, 1994). Cultivable microorganisms consisted mainly of aerobic species (Haveman et al., 2005). Microbial community comparisons of polluted and unpolluted aquifers have shown a higher diversity in the contaminated site (Cho & Kim, 2000; Roling et al., 2000). But there are also contradicting results from Reardon et al. (2004) who analysed bacterial communities from pristine and uranium-contaminated aquifer areas. He found that the majority of the detected phylotypes from both areas affiliated to Betaproteobacteria but with a higher level of diversity in the uncontaminated area. A comparison of the microbial community structure in surface water and associated groundwater displayed a higher diversity in the groundwater habitat, however the actual number of microorganisms was found to be lower (Kilb et al., 1998).

It has been demonstrated that ubiquitous microorganisms colonize the subsurface aquifer, establishing heterogeneous structures and compositions of the microbial community.

The differences depend on the heterogeneous conditions provided by the soil surroundings, water movement, and water composition of an individual site but also on other influences like irregular recharge with subsequent temporal changing of geochemical gradients (Haack et al., 2004b; Kolehmainen et al., 2007). In order to assess the microbiological quality of a given groundwater source it seems therefore necessary to analyse the specific situation of the site in question.

⇨ For contaminated aquifers, the development of microbial communitites depends on the type of contamination. Favourable substances (e.g. carbon sources) are usually characterized by high microbial numbers of little heterogeneity.

2.1.3 Association with clogging problems

Abstraction wells for drinking water supply are always linked to a given aquifer and its environment. Thus, the microbial flora present in groundwater (indigenous or anthropogenic introduced microorganisms) may also be found in the well water either as planktonic cells or as detached particles from biofilms or soil material. In addition, wells comprise a separate system with different specific parameters potentially important for microbial survival and multiplication, such as different materials to adhere, different nutritional and chemical water conditions, enhanced water flow, more space, and sufficient available oxygen concentration. This system has been extensively analysed by a number of authors (Cullimore et al., 2000; Hässelbarth & Lüdemann 1967).

Very often groundwater supplies measurable amounts of iron and manganese providing an energy source for iron- and manganese-oxidizing bacteria. These bacteria are ubiquitous. They occur in a variety of environments, also in groundwater, and are attached to soil, from where they enter into the well system. They catalyse the oxidation of ferrous iron (dissolved Fe (II) → Fe (III)) or manganese (dissolved Mn (II) → Mn (IV)) and deposit the minerals in structures outside their cells. Possible reactions are the following (Emerson & Moyer, 1997; Ghiorse, 1984):

$$2\ Fe^{2+} + ½\ O_2 + 5\ H_2O \rightarrow 2\ Fe(OH)_3 + 4\ H^+$$

$$Mn^{2+} + ½\ O_2 + H_2O \rightarrow MnO_2 + 2\ H^+$$

Fe (II) can also be subject of abiotic oxidation since it is thermodynamically unstable in the presence of oxygen at neutral pH (in acidic environments Fe (II) is stable) (Ralph & Stevenson, 1995). The oxidation rate however is usually too slow to account for the rapid accumulation of iron oxides in the well habitats.

Iron-oxidizing bacteria have been known for a long time, since they were easily detectable microscopically by their striking morphology. They include *Gallionella ferruginea* (Hallbeck et al., 1993; James & Ferris, 2004; Lütters-Czekalla, 1990), *Leptothrix ochracea* (Emerson & Weiss, 2004; Schieber & Glamoclija, 2007), and *Sphaerotilus*, which deposit iron in stalks or in sheaths outside their cell envelopes. The microorganisms *Crenothrix* and *Clonothrix* are often detected with Fe- or Mn-oxide encrusted filaments whereas the non-filamentous Siderocapsaceae species (*Arthrobacter* spp.) are surrounded by excreted extracellular capsular material (Chun et al., 2001). However, there are also Fe-oxidizing bacteria belonging to proteobacteria, which do not produce these morphological features, but are known to proliferate in microbial biofilms and deposit the Fe (III) oxides into an extracellular matrix (Emerson & Ghiorse, 1992; Emerson & Moyer, 1997; Nevin & Lovley, 2002; Roden et al., 2004).

Conditions favourable for the presence and multiplication of iron-bacteria are high concentrations of dissolved iron, the presence of at least low concentrations of dissolved oxygen, and increased water flow velocity (Sobolev & Roden, 2001; Stuetz & McLaughlan, 2004). They have been known to proliferate in waters containing

- dissolved iron concentrations in the range of >0.01- 2.15mg/L

- dissolved oxygen contents in the range of 0.1-10mg/L
- pH concentrations of 4.7-6.4 (Walter, 1997).

Cullimore & McCann (1978) reviewed iron bacteria in groundwater and wells. In Table 2 the occurrence of iron bacteria is shown and their ability to oxidize iron and manganese.

Due to their capability to excrete large amounts of polymeric substances together with mineral deposits they can readily accumulate thick layers of biofilm material which makes them one of the primary causes of severe clogging problems in wells and pore spaces of the surrounding aquifer, as well as in water distribution systems.

The effect of the growth of iron-oxidizing bacteria on water quality was analysed by Walter (1997). He analysed well water quality before and after reconditioning of the well (this included removing and steam-cleaning of the pump, adding sulfamic acid to the well to dissolve the biofilm, and subsequent pumping) and found that biofilms are capable of reducing the concentration of dissolved iron and manganese in the well water. This is consistent with the deposit of the minerals in the biofilm encrustations. Another statistically relevant effect was the increase of pH after reconditioning suggesting that during biofilm growth organic acids in the water are consumed or CO_2 is used as a carbon source and removed from solution.

Table 2: Range of habitats and ability of iron bacteria to oxidize reduced iron and manganese salts

Genus	Habitat				Oxidizes		
	Soil	**Fresh water**	**Mud sediments in lakes and rivers**	**Well water and piped systems**	**Acidic mine drainings**	**Fe only**	**Fe and/or Mn**
Sphaerotilus		x	x	x			x
Leptothrix		x	x	x	x		x
Crenothrix		x		x			x
Clonothrix		x		x			x
Gallionella	x	x	x	x		x	
Thiobacillus ferrooxidans	x	x	x	x	x	x	
Siderocapsa		x		x			x
Naumanniella	x	x		x			x
Ochrobium		x		x		x	

Adapted from Cullimore & McCann (1978).

An example for nutrient dependency is shown by Taylor et al. (1997) for a groundwater recovery well contaminated by organic compounds. They found in addition to iron oxidizing bacteria a consortia of sulphate reducers (*Desulfovibrio*), anaerobic (*Actinomyces*, *Bacteroides*, *Bacillus*, *Agrobacterium*) and aerobic heterotrophs (*Pseudomonas*, *Flavobacterium*, *Nocardia*, *Citrobacter*), iron-reducers (*Shewanella*), and sulphur-oxidizers (*Thiobacillus*).

An influence of the concentration of easily assimilable organic carbon (AOC) on clogging was found in an assay using a sand filter bed (Hijnen & van der Kooij, 1992). Acetate concentrations of 0.01mg C/L promoted clogging and suggested that the AOC concentration might be an important parameter for the clogging potential in the water.

In an assay mimicking biological clogging of aquifers, Dupin & McCarty (2000) found a preferential formation of aggregates and biofilm growth at neutral pH correlated with a decrease in conductivity of the water whereas increases in conductivity could be related to sloughing events.

- ⇨ Microbial induced clogging is usually due to iron oxidizing bacteria of different species,
- ⇨ Prerequisites are favourable ranges of iron, oxygen, pH and AOC.

2.2 Microbial biofilms

Microbial biofilms are ubiquitous and have been analysed in a variety of environments. Biofilms have been associated with medical devices, with drinking water supplies, with well clogging and biofouling processes. Adsorption of bacteria to solid surfaces results in the development of biofilms (Wimpenny et al., 2000). A biofilm is usually very heterogeneous and can be described as an assemblage of microbial cells irreversibly associated with a surface and enclosed in a matrix of extracellular polymeric substances (EPS). The matrix is produced by the bacteria themselves. It consists primarily of polysaccharides, but also of proteins and other polymers (Donlan, 2002). An example of a biofilm is shown in Figure 1:

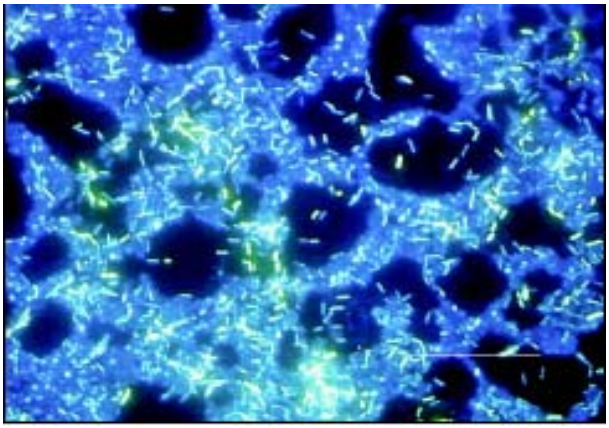

Figure 1: Polymicrobic biofilm (*Biofilm grown on a stainless steel surface in a laboratory potable water biofilm reactor for 14 days, then stained with 4,6-diamidino-2-phenylindole (DAPI) and examined by epifluorescence microscopy (Donlan, 2002). Bar: 20 µm.)*

Biofilm **development** is considered to start when various biotic and abiotic molecules from the water column (proteins, lipopolysaccharides, humic substances, mineral crystals, corrosion particles, clay particles) attach to available surfaces and build a first patchy monolayer together with microbial cells. Maturation to a multilayer biofilm proceeds with the adsorption of further microorganisms and their excretion of EPS. At the maturation stage, the microcolonies are encased in the matrix and separated from each other by small water channels allowing diffusion of nutrients and oxygen. Sequences in biofilm formation are shown in Figure 2 (Cullimore, 2000):

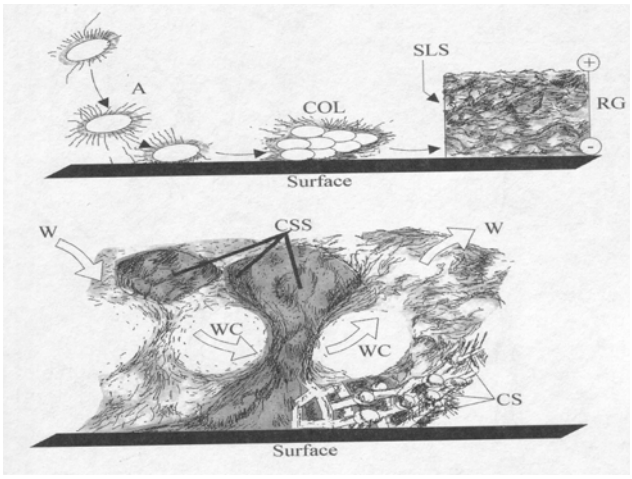

Figure 2: Biofilm maturation: A biofilm goes through a process of maturation from attachment (A) to colonization of the surface with a thin biofilm (COL), which grows into a series of simple layered structures (SLS), each operating on a separate part of the redox gradient (RG) through the biofilm. Finally, the biofilm mature into a complex set of slime (CSS) and crystalline structures (CS) that allow greater movement of water through conduits (WC) that pass through the structure and occupy as much as 40% of the volume.

Biofilm associated microorganisms display differences in their **metabolism** as compared to their suspended forms. Some genes are up-regulated in the attached state and some are down-regulated (Prigent-Combaret et al., 1999). The EPS are highly hydrated and may vary substantially in their chemical and physical properties, leading to characteristic properties of the biofilm (Sutherland, 2001).

Bacterial cells in a biofilm may be protected from the variable adverse conditions (nutrient shortage, predation, disinfection measurements) and find niches for persistence and growth by using specific diffusion gradients present in their microenvironment. Different surface materials have been analysed for their influence on cell attachment and biofilm formation (Schwartz et al., 1998), showing an enhanced colonization on PVC and polyethylene (see Figure 3):

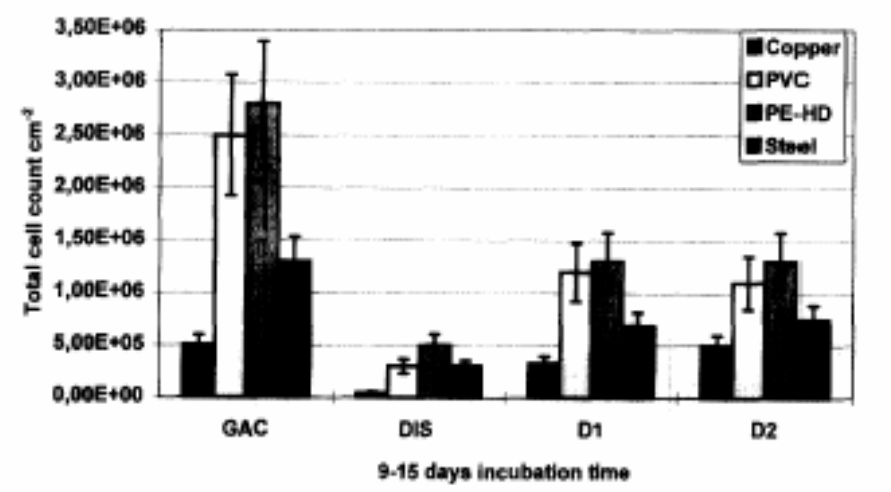

Figure 3: Biofilm formation on different pipe construction materials: The total bacterial cell count was evaluated by counting 10 microscopic ocular fields for each sample of test material in 2-3 independent experiments (GAC: granular activated carbon filtration; DIS: disinfection; D1, D2: two independent house branch connections; PE-HD: polyethylene).

In general, the formation of biofilms in the subsurface is dependent on the properties of the

- material available for attachment: surface structure and roughness, surface hydrophobicity
- hydrodynamics of the water: influence of flow velocity results in an equilibrium between attachment and detachment of cells in each biofilm, also determining the thickness of the biofilm (Bartetzko, 2002)
- water chemistry: water temperature, pH, concentrations of cations, nutrient content (Taylor et al., 1997)
- variable cell surface structures (fimbria, flagella), production of filaments, sheaths and stalks (especially from iron-oxidizing bacteria) of the microorganisms involved

Altogether, biofilm formation is not limited to the contribution of specific bacteria but depends on the above prerequisites permitting general microbial survival and growth in specific environments.

Bacterial pathogens have also been shown to be associated with biofilms (Camper et al., 1998; Hood et al., 1997). Although they have the capability to surface attachment, most of them are not able to grow extensively under these conditions due to specific nutrient or other requirements for growth. Nevertheless, the existence of a biofilm always includes the risk of accumulated pathogenic microorganisms, which may detach at times and provide a risk for human health.

⇨ Biofilms usually consist of non-pathogenic bacteria in a matrix of EPS. However, biofilms may accumulate pathogens due to increased surface attachment.

2.3 Microbial pathogens

Originally, groundwater was thought to be free of pathogens. However, waterborne disease outbreaks associated with systems using groundwater sources were frequently reported since surveillance systems (US) had been established in 1971 (Barwick et al., 2000; Blackburn et al., 2004; Craun, 1984; Craun & Frost, 2002; Kramer et al., 1996; Lee et al., 2002; Liang et al., 2006; Zimmerman & Lindsay, 2006; Hrudey & Hrudey, 2007).

Contaminated groundwater, faulty well construction, improper well location or maintenance has been attributed to such events. Detailed analyses and advanced detection methods led to the identification of the etiological agents responsible for the infections and proved the occurrence of a variety of pathogenic and non-pathogenic bacteria, viruses, and protozoa in groundwater and connected wells (Bauder et al., 1991; Leclerc et al., 2002; Macler & Merkle, 2000).

It is generally assumed that the occurrence of pathogenic bacteria in groundwater and wells most likely results from human or animal faecal contamination introduced into the subsurface. Main components from this contamination are represented by the Enterobacteriaceae. Whereas most members of this family are normal inhabitants of the gut of mammals and not pathogenic there are enteropathogenic species of *E. coli* (e.g. strain O157/H7) (Chalmers et al., 2000; Jackson et al., 1998; Swerdlow et al., 1992), *Salmonella*, *Shigella*, *Yersinia* (Schiemann, 1990), and other species like the proteobacterium *Campylobacter* (Alary et al., 1990; Buswell et al., 1998; Clark et al., 2003; Megraud & Serceau, 1990; Millson et al., 1991; Rollins & Colwell, 1986; Stanley et al., 1998), and Vibrio (Cavalieri d'Oro et al., 1999; Clark et al., 1998), which are connected to serious health concerns.

Table 3 gives an overview of the pathogenic microorganisms (bacteria, viruses, protozoa) in groundwater associated with human disease outbreaks.

Enteric viruses are also introduced into the water system via humans and animals and may remain infectious in soil and water for prolonged times (depending on the heterogeneous conditions of the site). A number of virus types are water-related pathogens, including enteroviruses (coxsackievirus, echovirus), rotavirus, adeno- and norovirus, hepatitis A and E virus (Fong & Lipp, 2005). They may constitute major sources of infection (Abbaszadegan et al., 1999; Botzenhart, 2007; Frost et al., 2002; Herwaldt et al., 1992; Lawson et al., 1991; Leclerc et al., 2002) in particular with regard to the low infectious dose for some viruses (e.g. Norovirus).

Pathogenic protozoa, like *Cryptosporidium parvum* and *Giardia lamblia*, are usually linked to an influence of surface water on groundwater (Craun, 1991; Craun et al., 1998; Goldstein et al., 1996).

The occurrence of pathogenic microorganisms, their concentration, the conditions for their survival or even growth in the well, and especially their capacity for persistence in biofilms are important parameters for well water quality.

Table 3: Microbial pathogens in groundwater associated with disease outbreaks

Bacteria (size: 1-10 µm)	**Infectious dose**	***Incubation period**	**References**
pathogenic *E. coli*	10-100	12-72 hours	Rice (1999); Tschäppe (2000)
Salmonella spp. typhoid Salmonella enteric Salmonella	10^1-10^5 10^2-10^3 10^5-10^6	 1-3 days 6-72 hours	Covert (1999); Tschäppe (2000)
Shigella spp.	10-100	1-7 days	Barwick et al. (2000); Maurer and Stürchler, (2000)
Yersinia enterocolitica	10^4-10^9	1-7 days	Schiemann (1990); Fricker (1999); Tschäppe (2000)
Legionella spp.			
Campylobacter jejuni	10-500	1-7 days	Tschäppe (2000)
Vibrio cholerae	10^6-10^8		Ford (1999)
Viruses (size: 0.02-0.08 µm)			
Enteroviruses: Poliovirus Coxsackievirus A and B Echovirus Enterovirus	5-10		Metzler et al. (1996), Walter (1999), Gerba (1999)
Norovirus (Norwalk-like virus)	1	12-48 hours	Gerba and Rose (1990), Kukkula et al. (1999)
Rotavirus	10-100		Metzler et al. (1996), Botzenhardt (2000), Abbaszadegan (1999)
Hepatitis A and E	1-10	15-45 days	Walter (1999), Sobsey (1999)
Adenovirus			
Protozoa (size: 5-20 µm)			
Cryptosporidium parvum	1-30 oocysts	appr. 7 days	Metzler et al. (1996), MacKenzie et al. (1994), Ford (1999); Carmena et al. (2007)
Giardia lamblia	1-10 cysts	7-14 days	Metzler et al. (1996), Gornick et al. (2001); Carmena et al. (2007)

Adapted from Auckenthaler & Huggenberger (2003); Botzenhart (2007); *Craun (1984).

⇨ A wide variety of pathogenic bacteria, viruses and protozoa occur in groundwater.
⇨ These micro-organisms are the result of human or animal faecal contamination.

2.4 Indicator microorganisms

Pathogens usually are not present in the subsurface environment in adequate numbers to be easily detected. Since the water cannot be tested for all possible pathogens due to their large variety, specific bacteria have been selected as indicators of faecal contamination of drinking or water (German drinking water ordinance - Trinkwasserverordnung, 2001). They are used to alert for the presence of a microbiological contamination from faecal origin, which might represent the causative agents for water-borne illnesses.

Indicator bacteria are expected to exhibit the following characteristics:

- to be a substantial part of the intestinal flora of mammals,
- to be present when pathogens are present, but not to be pathogenic themselves,
- to be present in greater numbers,
- to survive and resist detrimental environmental conditions or disinfection in a comparable manner to pathogens,
- not to grow in the environment,
- to be detectable with sensitive (even in very small numbers), specific (detection of only the target organism), rapid, easily performable, and low cost methods (Bitton, 2005; National Research Council, 2004)

Widely used indicator bacteria for faecal pollution of drinking water have been the coliform group of bacteria, the faecal coliforms, which are the thermotolerant members of the coliforms, including *E. coli*, in addition enterococci and sulfite-reducing clostridia (Atherholt et al., 2003; Craun et al., 1997; Leclerc et al. 2001; Yates, 2007). Table 4 gives an overview on the occurrence of some bacterial species in faeces and their use as indicators:

Table 4: Faecal intestinal flora studies

	US individuals[1]		European adults[2]	
	% positive	mean range	% positive	mean range
Bacteroides	100	11.3	100	9.6
Eubacterium	95	10.6	--	--
Bifidobacterium	100	10.2	79	8
Lactobacillus	79	10.4	100	9
**Clostridium*	73	9.3	100	8.3[3]
**Enterococcus and Streptococcus*	100	9.1	100	7.2
**Escherichia coli*	94	8.8	100	7.8
**Citrobacter*	10	7.5	70	3.3
**Klebsiella*	24	6.9	48	2.4
**Enterobacter*	13	7.5	9	--

[1]From Finegold et al (1983), [2]Leclerc & Moriamez (1980): range and mean count of bacteria expressed as number of organisms log10 per gram faeces (dry weight)[1] and (wet weight)[2]. Clostridium spores[3]. *bacteria commonly used as indicators of faecal pollution.

Coliform bacteria are rod-shaped bacteria, aerobic, facultative anaerobic, gram-negative, oxidase-negative, do not form spores, are able to grow in the presence of bile salts and can ferment lactose, with the production of acid and gas within 24-48 hours (WHO, 1996). The differentiation to other environmental bacteria is based on their metabolic capacity to ferment lactose. The cleavage of this disaccharide with the enzyme ß-galactosidase cannot be performed by the vast majority of other soil and water bacteria.

Faecal coliform bacteria are members of the total coliforms. Moreover, they are inhabitants of the intestinal tract of mammals, which makes them thermotolerant. They are able to ferment lactose at a higher temperature. They include members of the family Enterobacteriaceae with the genera *Escherichia*, *Klebsiella*, *Enterobacter*, and *Citrobacter*. Faecal coliform bacteria are present in high numbers in human (and animal) faeces.

E. coli account for approximately 95% of the coliform flora in human faeces (in concentrations of 10^8-10^9/g faeces) whereas *Klebsiella*, *Enterobacter*, and *Citrobacter* species predominate in sewage (Leclerc et al., 2001). Differentiation between the coliforms and *E. coli* are achieved by incubation at different temperatures and by growth on specific culture media (see Trinkwasserverordnung, 2001). Generally, it is agreed upon that coliforms can also be found in soil and fresh water environments, whereas *E. coli* is almost exclusively found in faeces and does not multiply appreciably in the environment. Its presence is therefore taken as a strong indication of faecal contamination.

Analyses in tropical environments however have provided evidence for an extraenteral existence of *E. coli*, which has to be taken into account when *E. coli* is used as indicator in the tropics (Bermudez & Hazen, 1988; Lopez-Torres et al., 1987; Rivera et al., 1988).

Enterococci include the faecal streptococci and among them essentially the species *E. durans*, *E. faecalis*, *E. faecium*, *E. hirae* which originate from the gut of humans and animals. They rarely multiply in the environment, are reported to persist for longer periods, and have been shown to be more resistant to environmental stress than coliforms (Bitton et al., 1983; John & Rose, 2005; US EPA, 2000).

According to the German drinking water ordinance, *E. coli* and Enterococci should not be detectable in 250 ml of drinking water. An overstepping of this threshold results in severe measures to avert health risks. For coliform bacteria, the same detection limits are valid, however measures after a positive finding are less strict. This implies that a detection of coliforms may not always be an indication of a health risk, because they could also arise from non-faecal origin (Feuerpfeil & Szewzyk, 2003).

The spores of ***Clostridium perfringens*** are used as indicators of water-transmitted protozoa (Brookes et al., 2005). Especially in cases where suspicion emerges of surface water contaminating drinking water, the monitoring is recommended to apply additional tests for the detection of clostridia. They are gram-positive, rod-shaped bacteria belonging to the family of Clostridiaceae, they grow strictly anaerobic, use a fermentative metabolism, and produce heat-resistant endospores, which can survive harsh environmental conditions or disinfection procedures and start re-growth if conditions are favourable. These spores are very robust and may serve as an indication for a possible but not necessarily a recent introduction of contamination, because of their long persistence in the environment as compared to pathogens.

Recently, ***Bacteroides*** have been suggested for use as indicators of faecal pollution as well. They are highly abundant in faeces and some strains seem to show host specificity. Since traditional culturing methods do not produce the necessary degree of specificity, the detection of these bacteria relies on molecular techniques. Different genetic markers have been identified which can be used to detect host-specific faecal contamination (Ahmed et al., 2008; Layton et al., 2006; Savichtcheva et al., 2007). The use of bacteriophages infecting Bacteroides as additional or alternative indicators (because of their potential human specificity) is investigated and discussed by a number of authors (Blanch et al., 2004; Ebdon et al., 2007; McLaughlin & Rose, 2006).

The use of **bifidobacteria** as faecal pollution indicators has been proposed on the findings that they are also present in high numbers in human faeces (see Table 4).

Since they are obligate anaerobes the appropriate culturing methods are difficult and elaborate and have been found to be of limited selectivity (Rhodes & Kator, 1999). Hence detection is primarily based on molecular detection methods (Bonjoch et al., 2004).

Somatic and F-specific coliphages have widely been used as indicators for the presence of pathogenic viruses (Atherholt et al., 2003; LeChevallier et al., 2006; Leclerc et al., 2000; Plummer & Long, 2007). Whereas in Berlin bacteriophages are not routinely investigated, e.g. in The Netherlands bacteriophage tests are integrated as part of routine water quality analyses.

Coincidence of indicator and pathogen

The question if indicator microorganisms can mimic to a certain extent the presence of physiologically similar pathogenic enterobacteria has been the target of numerous analyses. Differential persistence and survival rates have been demonstrated for the different indicator organisms in environmental waters (Anderson et al., 2005; Deller et al., 2006; Havelaar et al., 1993; Noble et al., 2004) showing that survival is a combined function of physical, chemical, and biological factors (Astroem et al., 2007; Rhodes & Kator, 1988).

Van Lieverloo et al. (2007) found a high variability in the coincidence of pathogens and thermotolerant coliforms in environmental samples and thus questioned the applicability of the indicator principle for assessing infection risks. Korhonen et al. (1996) detected *Listeria monocytogenes* and *Yersinia enterocolitica* in wells where no coliforms could be detected. As there is obviously not a single indicator organism which can reasonably be used for the prediction of the presence of all pathogens, Touron et al. (2007) suggested therefore that the reliability of a correlation between indicator bacteria and a putative pathogen should be assessed for each environment under investigation.

Survival and inactivation rates in groundwater were found to be similar for coliphages, poliovirus, echovirus, coliform bacteria, enterococci and *Salmonella* spp. whereas those for hepatitis A virus and coxsackievirus were lower (John & Rose, 2005). A number of authors have published different inactivation rankings for indicator microorganisms and phages (Duran et al., 2002; Noble et al., 2004) showing that the inactivation process is highly system specific.

Despite the long use of *E. coli* and coliform bacteria as markers for faecal contamination and indicators of pathogens in water samples there is increasing evidence that these microorganisms do not correlate well with non-bacterial pathogens like protozoa or viruses (Duran et al., 2002; Tallon et al., 2005; Payment et al., 2003). Leclerc et al. (2001) reviewed the suitability of coliform bacteria as markers of microbial water safety and concluded that their fate does not reflect the elimination of enteric viruses and protozoa. This is in agreement to epidemiologic data of Craun et al. (1997) suggesting that the determination of coliform bacteria is not adequate as marker for protozoan contamination**,** because coliforms are less resistant to environmental stresses and disinfection (Medema et al., 1997).

It has also been shown that well water could contain human enteric viruses without any faecal pollution indicators. This suggests a limited value of these bacteria in predicting the presence of virus due to their different responses to environmental stress, their adsorption capacities, survival, and inactivation behaviour (Locas et al., 2007). Bacteriophages have been introduced to serve as indicators for the presence of pathogenic viruses and somatic and F-specific coliphages have been widely tested for this purpose. The potential of bacteriophages is based on their similarities in size and morphology, inactivation and sorption characteristics, and on their non pathogenicity. However, bacteriophages cannot consistently be recovered from faeces and only approximately 3% of humans carry F^+ phages (Leclerc et al., 2000).

In epidemiological examinations, usually no association between the detection of bacteriophages and disease outbreaks could be established. The value of bacteriophages as indicator organisms is presently discussed in terms of a correlated incidence of phage and pathogen virus, easy and fast detection and quantification (Collins et al., 2006; Ibarluzea, et al., 2007; Mandilara et al., 2006; Lucena et al., 2006).

- ⇨ Indicator organisms like E.coli or Entorococci are widely used to identify faecal contamination.
- ⇨ Their presence, however, does not always correlate with pathogenic micro-organisms, especially with the occurrence of virus contaminations.
- ⇨ New indicators for virus contamination are currently being discussed (e.g. bacteriophages).

Chapter 3 Sources of microbial pollution

Microbial contamination of groundwater and well water is generally considered to occur by introduction of faecal pollutants from human or animal origin into the subsurface. The contaminants are transported with the water into the aquifer and into the wells. Thus recharge water on the one hand is the basis of the transport and on the other hand it might itself represent a source of contamination depending on its nature and origin.

3.1 Recharge water

The predominant natural recharge source for groundwater is precipitation. Rainwater can provide rapid transport pathways through the unsaturated zone by the downward water flow taking faecal contaminants along with it. Depending on the chemical composition, the abundance and infiltration rate of the water involved, bacteria and viruses present at the surface and within the soil may be detached from the solids and mobilized by the enhanced water flow, thereby increasing microbial contamination of the subsurface (Keswick, 1984). Bacterial contamination has been reported to coincide with heavy rainfall (Astroem et al., 2007; Millson et al., 1991). Recharge is known to induce chemical and nutritional gradients leading to temporal changes in community structure (Haak et al., 2004b).

In urban environments, direct precipitation might be locally reduced, but water runoff from sealed surfaces is enhanced thus providing rapid pathways into surface waters.

In addition, further recharge for rural aquifers occurs through infiltration of surface waters from rivers, lakes, and artificial infiltration areas like ponds and wetlands (Barrett et al., 1999). The extent of the microbiological loads in these water sources determines to a certain extent the pollution of the subsurface although retention by filtration processes in the soil and die-off diminish the impact.

In general, the quality of the recharge water, its abundance and rate of infiltration, as well as the location of infiltration and the distance to the discharge site should be assessed in contamination events.

⇨ Precipitation (natural recharge), or infiltrating surface waters (bank filtration, aquifer recharge) act as pathways for faecal contamination of groundwater or may mobilize previously attached micro-organisms in case of sudden flow rate changes.

3.2 Faecal pollution sources

The introduction of faecal pollutants into the subsurface may occur from various sources of human and animal disposal activities in different concentrations, forms, and severity of impact. Keswick (1984) has identified major sources of microbial groundwater pollution and in parallel given an evaluation of their importance for putative contamination events (see Table 5).

They comprise contamination from municipal sources like liquid sewage, sewage sludge, and solid wastes, from the agricultural sector with animal wastes, as well as from polluted precipitation run-off and surface water.

The potential for groundwater pollution derives from the various disposal methods of these wastes and from unintentional leakage of disposal units.

Land disposal of sewage. Disposal of sewage, sewage sludge, and solid wastes by land application through slow-rate or rapid infiltration, wetlands, or subsurface injection may lead to contamination of the subsurface. Usually these methods are only applied in areas where the impact on groundwater is negligible due to a favourable geologic situation or low probability of human contact.

Table 5: Major sources of groundwater pollution

Source	Evaluation of microbial influence
municipal:	
sewer leakage	primary
sewage effluent	primary
sewage sludge	primary
solid wastes	secondary
agricultural:	
animal wastes (feedlots and dairies)	primary
stockpiles	variable
miscellaneous:	
polluted precipitation and surface water	variable
septic tanks	primary

Adapted from Keswick (1984) in: Bitton & Gerba (eds.) Groundwater pollution microbiology

Impact of leakage from sewers, septic tanks, on-site facilities. Facilities for the containment of sewage and the pipelines to the discharge sites may not be properly sealed and thus often represent direct sources of faecal contamination in the subsurface. Frequently reported pollution sources were leakage of contaminants from sewers (Abbaszadegan et al., 2003; Barrett et al., 1999; Collins et al., 2006; Harvey et al., 1984; Paul et al., 2004; Scandura & Sobsey, 1997; Verstraeten et al., 2005). The detection of coliform bacteria correlated with the presence of a septic system near the sampling site, but was also found to be dependent on well depth (Francy et al., 2000). Fong et al. (2007) reported a massive groundwater contamination caused by influx from wastewater treatment facilities and septic tanks to the subterranean water after extreme precipitation resulting in a more or less unrestricted influx of contaminants into the wells.

Impact of agriculture on groundwater microbial pollution. Microbial contamination of domestic wells has been described as a common problem in many rural areas (Fitzgerald et al., 2001; Mroz & Pillai, 1994). It has been attributed to anthropogenic manipulations such as irrigation and manure spreading (Lerner et al., 1990). Different farming practices (tillage practices and different manure managements) are reported to have a strong impact on the quality of recharged groundwater systems (Celico et al., 2004; Cho et al., 2000; Cho & Kim, 2000; Entry & Farmer, 2001; Kulabako et al., 2007; Thiagarajan et al., 2007). High bacterial concentrations have been related to intensive agricultural land use (Zimmerman & Lindsay, 2006) and to daily manure spreading (Conboy & Goss, 2000).

Impact of surface water. In areas where surface water is microbial polluted and where a rapid influx into the groundwater without proper retention can occur, enhanced possibilities of contamination result. Well water contamination was described by Lamka et al. (1980) to be caused by the influence of polluted surface waters. Virus contamination could be attributed to a surface water contribution to well water, too (Borchardt et al., 2004). DaFranca et al. (2006) analysed the occurrence of bacterial contamination in a well field where part of the groundwater was derived from a near contaminated creek. They could show that transit times of bacteria from the creek to the wells were in a time range, which made the detected contamination of the wells plausible.

Impact of other pollution sources. Direct pollution of wells might arise from the use of contaminated materials during construction, maintenance or monitoring procedures by humans (Gelinas et al., 1996; Conboy & Goss, 1999).

A number of case studies concerned with well water and groundwater contamination is listed in Table 6:

Table 6: Reported cases of faecal contamination sources in wells

Object and site	**Country**	**Investigated microorganisms**	**Putative or identified sources of contamination**	**References**
wells	North-West England	Cryptosporidia	contamination from surface runoff (grazing pasture) during heavy rainfall	Bridgman et al., 1995
rural drinking water wells	Canada, Ontario	coliforms, Streptococci, *Clostridium perfringens*	high risk wells correlated to manure spreading on farms (faecal Streptococci, *Clostridium*), to age of the wells, to dug or bored wells	Conboy & Goss, 1999, 2000
boreholes	Korea, Wonju	coliforms, faecal Streptococci, Clostridia	contamination correlated to infiltrated livestock wastewater	Cho et al., 2000
on-farm groundwater supplies	Canada, Alberta	coliforms	correlation between total coliforms, primary agriculture and shallow wells	Fitzgerald et al., 2001
springs in unconfined aquifer	southern Italy	faecal Enterococci	contamination correlated to precipitation events and cattle grazing	Celico et al., 2004
wells		coliforms	correlation of higher bacterial concentrations to higher percentages of agricultural land use	Zimmerman & Lindsay, 2006
community and non-community wells	US	total coliforms, *E. coli*	significant correlation between total coliforms, aquifer type, agricultural land use, and the existence of septic systems on the well premises	Francy et al., 2000
public water supply		Shigella	on-site soil disposal systems	Weissman et al., 1976
groundwater wells	US, N.Y. Nassau County	enteric viruses	virus detection correlated to shallow site, near sewage recharge basins (ca. 10.4 m above the aquifer)	Vaughn et al., 1978
municipal well sites	US, Wisconsin	Enterovirus, Rotavirus, Hepatitis A, Norovirus	no correlation to surface water infiltration or to faecal indicators, no seasonality. Most likely source were municipal sanitary sewer lines	Borchardt et al., 2004
wells	Germany, Rastatt	coliforms and *E. coli*	contamination inversely correlated to distance of sampling to leaky sewage facilities	Paul et al., 2004
groundwater wells	US, Lake Erie, Ohio	coliforms, Enterococci, enteric viruses	contamination by rapid mixing of surface water (influenced by wastewater treatment facilities) and groundwater	Fong et al., 2007
domestic shallow sand-point and cased wells	US, Nebraska	male-specific coliphages	sand-point wells more susceptible to coliphage contamination (resulting from septic waste) than cased wells, distance to pollution source important (< 15 m deep)	Verstraeten et al., 2005
wells	Brazil	coliforms	contamination of wells correlated to river water contribution	Da Franca et al., 2006

rural wells	Zimbabwe	coliforms, Streptococci, *Clostridium perfringens*	pollution correlated to unprotected well heads, resulting from the deposit of manure in the immediate vicinity of the wells	Conboy & Goss, 1999
private wells in rural community	US, Oregon	coliforms	contamination correlated to rainfall, reflecting a leakage from surface water into improperly sealed wells	Lamka et al., 1980
wells	US, Texas	faecal coliforms	surface water as possible pollution source	Mroz & Pillai, 1994
municipal well, nonresidential area	US, Wisconsin	Enterovirus, faecal indicator organisms	correlated to infiltration of river water; infiltration measured by determining hydrogen and oxygen isotope ratios (18O/16O, 2H/1H)	Borchardt et al., 2004
wells	Walkerton, Ontario, USA	E. coli O157:H7, Campylobacter jejuni	contamination correlated to heavy rainfall and manure spreading near the well	O'Connor, 2000; Holme, 2003

The sources of contamination are variable and it seems most important to minimize as far as possible the pollution of the water catchment area for the groundwater/well system, and to ensure a contamination free operation and monitoring procedure by appropriate handling of the well equipment.

- ⇨ Sources of faecal contamination are sewage and manure, that are either directly spread on agricultural fields or affect infiltrating surface water.
- ⇨ Heavy rain events and unprotected well-heads often correlate with microbial contamination.

Chapter 4 Microbial pathways into the subsurface

Microorganisms may be introduced into the groundwater and well environment either

- by passive transport with water from the surface
- by migration with the direction and flow rate of the groundwater system
- by direct deposition of polluted material into the subsurface

In the following, geographical and geological factors influencing microbial pathways into the subsurface will be described and discussed in terms of their potential to affect well water contamination. In addition, factors are analysed which affect the movement and retention, the survival and inactivation of microorganisms during their pathway from top to the subsurface. Specific variables provided by the participating microorganisms are also considered.

4.1 Geographical factors

Landscape characteristics and the relative location of the well within that geographic area might influence the susceptibility of wells to contamination (Gosselin et al., 1997). The well site might be near or quite far away from the site of polluted recharge depending on local, intermediate, and/or regional flow systems present in that landscape. All three flow system types might contribute to contamination.

⇨ If a well is situated downstream of a pollution source, it is susceptible to contamination,
⇨ The risk for contamination decreases with rising distance from the contamination source

4.2 Aquifer-specific geology

Groundwater flow systems differ in their hydraulic conductivity, in pore sizes, and in the establishment of hydraulic gradients. These are important parameters for the determination of volumes and rates of groundwater movement (USGS circular 1224, 2002). Low conductivity is found in **clay, silt, and loess** with small pore sizes through which organic material and particles cannot readily penetrate. This results in shortage of nutrients in the subsurface and in low or no transport of microorganisms.

Aquifers situated in areas of **sand and gravel** originating from streams and rivers however are often in contact with surface waters, resulting in sufficient nutrient supply for microorganisms, thus promoting microbial colonization. Such an environment may pose a pollution risk for the well water.

Consistently higher numbers of coliforms and faecal coliforms have been reported in **basalt** aquifers (with high velocity flow) than in sand aquifers (slow water movement) (Entry & Farmer, 2001). The enhanced flow velocity results in reduced sorption of microorganisms to sediment surfaces because of a reduced likelihood of contact.

Aquifers in **sandstone and gneiss** environments possess various crevices and fissures, allowing moderate nutrient supply. In this environment, different areas are sometimes not connected to each other and therefore differential microbial populations frequent.

Karsts are calciferous bedrocks with strong decomposition, exhibiting large canals and crevices through which transport of water and nutrients may be rapid from soil top to subsurface rendering the subsurface extremely susceptible to contaminants. Here bacterial concentrations show a rapid response to changes in the conditions of surface and irrigation water within the recharge area (Mahler et al., 2000).

Sites with older limestone or dolostone bedrock, clay or clay loam soil were identified as high-risk elements for the susceptibility of wells to contamination (Conboy & Goss, 2000).

Another important factor is the depth of an aquifer. A shallow aquifer tends to be more vulnerable to contamination than a deep one.

Thus, the direction, the length, and migration time of the groundwater flows and the hydrological linkage to adjacent aquifers and surface waters influence the transport of microbes into the subsurface.

- ⇨ Wells located in fractured rocks and karst aquifers show highest risk of microbial pollution.
- ⇨ The risk of contamination decreases with rising depth of the well.

4.3 Interaction with the soil and aquifer matrix

The pathways of microorganisms moving with the water into the subsurface are influenced by a variety of factors among which the composition of the surrounding soil, the nature of minerals present, the extent of the saturated and unsaturated zones play a major role. Soil properties are responsible for the moisture-holding capacity, the pH, and the trapping of organic matter. These properties influence the survival of microorganisms whereas predominantly particle and pore sizes, ionic strength, and clay content determine their retention (Fontes et al., 1991).

In the unsaturated zone, a minimum of moisture on the surfaces of soil particles is required along which microorganisms may migrate. Here they come into close contact with the surfaces resulting in good adsorption possibilities. This limits microbial travel time and distance. A greater speed and travel distance is possible during the movement through the saturated zones of the aquifer leading to a less efficient retention of the microorganisms. During rainfall a lowering of ionic strength and salt content of the pore water may cause a more rapid transport as well as an enhanced desorption of pathogens from solids leading to further migration into the subsurface.

Physical filtration (removal of particles from solution by deposition on porous media) and **straining** (trapping of particles in pores too small to allow passage) are the main limitations for the movement of bacteria through soil (Rusciano & Obropta, 2007; Stevik et al., 2004). The pore sizes of sand and silt are approximately similar to the size of bacteria and correspondingly the transport of bacteria is reduced whereas in coarser materials with larger pore sizes the transport is faster (Huggenberger, 2003). Bacteriophages are transported faster through soil whereas faecal coliforms travel more slowly and only lower numbers can be detected in the leachate (McLeod et al., 2004). This is possibly an effect of the much smaller size of the phages and their different adsorption and inactivation rates.

The reduction of microorganisms by filtration depends not only on the geological materials present in the path to the subsurface, but also on the speed, duration, and distance of their movement (Hagedorn, 1984; Lundh et al., 2007; Logsdon et al., 2002; Pang et al., 2003). As an example, riverbank filtration has been reported to affect water quality positively by removing suspended solids, chemical contaminants, organic carbon, and pathogens (Kuehn & Mueller, 2000; Tufenkji et al., 2002).

A number of transport distances for microorganisms has been listed in Table 7, which show their dependence on the different geological materials. However, It must be noted that in many examples details of hydraulic conductivity and aquifer flow have not been described.

During their path through the soil and groundwater system microorganisms are subject to dispersion, a mechanism, which leads to a reduction of peak concentrations and an increase in peak width (Bales et al., 1989; Foppen et al., 2007).

Diffusion usually accounts for minor movements and is regarded negligible. In addition, microorganisms are subject to **convective transport** and connected to the movement and velocity of pore water, which in turn is governed by hydraulic pressure gradients, porosity and permeability distribution of the soil in that environment (Ginn et al., 2002).

Usually, tracer experiments are applied in examinations of groundwater flow and transport for an assessment of groundwater and well susceptibility to contamination. In addition to chemical tracers, bacteriophages have also been used because they are non-pathogenic and easy to detect and differentiate from the indigenous microflora. It has been shown that the transport of bacteriophages may occur much faster than the average velocity of the groundwater flow (Taylor et al., 2004). A faster travel speed than that of chemical tracers has also been reported for Cryptosporidia oocysts (small reproduction cells) in sandy soils and aquifer sediments (Harter et al., 2000). This is due to the fact that the transport of the micro-organisms is limited to large pores, whereas chemical tracers migrate through all sizes of pores that contribute to groundwater flow. However, the rapid velocity flow, carrying the particles, commonly represents only a minor portion of the average water flow velocity but is an important factor to be considered. It should also be kept in mind that usually only a part of the microbial tracers are recovered, the rest may be held back by filtration or adsorption, dispersion in the subsurface, or by inactivation.

The **retention** efficiency for *E. coli* in planted and unplanted subsurface wetlands were analysed, but no significant difference in removal was found (Sleytr et al., 2007; Tietz et al., 2007).

The diversity and overall concentration of microorganisms decrease with depth depending on the length and speed of travel (Celico et al., 2004). This effect does not occur equally distributed, but depends on the compartmentation and heterogeneity of the surrounding geological situation with different densities and adhesion surfaces (Gerba & Bitton, 1984; McKay et al., 1993; Mueller, 1996). The overall gradual reduction of concentration with depth holds also for suspended organic substances.

Specific rock surfaces and soil particles present in the pathway from top to the subsurface might provide favourable attachment and survival conditions, since it has been shown that mineral inclusions can be exploited by bacteria as nutrient sources (Rogers, 1998; Rogers, 2002; Rogers & Bennett, 2004). In fact, microorganisms might persevere in specific niches where conditions for permanent colonization and multiplication are favourable. Such zones thus represent preferential habitats for distinct microbial communities and might provide permanent sources of microbiological influx into the well surroundings.

- ⇨ Concentrations of micro-organism are reduced in the subsurface due to inactivation and adsorption.
- ⇨ Maximum travel distances vary greatly (< 1 m to > 1000 m), depending on micro-organism and substrate.
- ⇨ Transport of micro-organisms occurs through the large pores, thus they may show higher transport velocities than chemical tracers.

Table 7: Transport distances of microorganisms derived from field experiments

Microorganism	Maximum travel distance (m)	Travel time	Hydraulic conductivity (m/d)	Mean pore velocity (m/d)	Colloid velocity (m/d)	Aquifer material, thickness	References
Bacillus sterothermophilus	900		*10^4	164	200	gravel	Martin & Noonan, 1977
Bacillus sterothermophilus	28.7	24-30 h				crystalline bedrock	Allen & Morrison, 1973
Coliforms	350-830		*10^5	160		sand with gravel, rubble, 4-8m	Anan'ev & Demin, 1971
fecal coliforms and streptococci	457	15 days				coarse gravels	Merrell et al., 1967
Coliforms	30.5	35 h				sand and pea gravel aquifer	Krone et al., 1958
fecal coliforms	9.1					fine loamy sand to gravel	Bouwer et al., 1974
Coliforms	6.1					fine to medium sand	Young, 1973
Coliforms	0.6-4					fine sandy loam	Butler et al., 1954
Phage T4	1600					carbonate rock	Fletcher & Myers (1974)
Phage T4, ΦX174	920					gravel	Noonan & McNabb (1979)
Coxsackie B3 and unidentified viruses	408					coarse sand with fine gravel	Vaughn & Landry (1977)
phages	400		4.6-19.5	3-12		fine sand with gravel, coarse sand	Aulenbach (1979)
Poliovirus, Coxsackie B3, Echovirus	250					sand with coarse gravel	Koerner & Haws (1979)
Coliphage f2, enteroviruses, faecal streptococci	183		8.6			silty sand and gravel	Schaub & Sorber (1977)
Echovirus 6, 21, 24, 25 and unidentified bacteria	45.7					coarse sand with fine gravel, 1-2% silt	Vaughn & Landry (1977)

Adapted from Rehmann et al. (1999); Huggenberger (2003); Hagedorn (1984).

* order of magnitude estimated from given velocity and hydraulic gradient values.

4.4 Factors and processes influencing the survival of micro-organisms

For the survival and persistence of microorganisms in the subsurface essentially two main processes have to be considered. These are the adsorption to particles and mineral surfaces present in the subsurface and the inactivation during their movement. Numerous analyses have been undertaken to determine the influencing factors present in different environments. In the following major factors are presented which are responsible for either microbial inactivation or adsorption or for both processes leading to the removal of microbial contamination.

Adsorption is a complex process influenced by different characteristics of the participants, such as water, surface material of soil particles and type of microorganism (Donlan, 2002). Table 8 gives an overview on the variables of bacterial attachment:

Table 8: Variables affecting attachment of pathogens during their path into the subsurface

Factor	**Influence**
Aquifer or soil texture	Fine-textured substrates retain viruses, bacteria and protozoa better, due to increased interaction and adsorption. Fractured rocks are poor retainers of microorganisms.
Water flow	Driving force of transport. Increased water flow reduces adsorption and may remobilize adsorbed microorganisms.
pH	Low pH increases adsorption of microorganisms to soil particles, due to reduced electrostatic repulsion.
Cations	Presence of multivalent cations increases adsorption, due to the formation of salt bridges between negatively charged microorganisms and soil particles
Metal hydroxides	Iron hydroxides improve adsorption of microorganisms
Soluble organics	Different influences: better adsorption due to preconditioning surfaces with protein molecules, on the other hand: competition of organic matter to sorption sites increases movement through the subsurface
Microorganism characteristics	Bacteria and protozoa are more readily removed than viruses, due to their smaller size, differences in isoelectric points and surface composition determine rate of adsorption.
Saturated versus unsaturated flow	Under unsaturated flow conditions, water fills only the small pores, which increases the contact time and thus increases adsorption.

Adapted from Gerba & Bitton (1984); Schijven & Hassanizadeh (2000); WHO report (2003).

Under oligotrophic conditions, the majority of bacteria are usually attached to solid particles in the different zones of an aquifer (Harvey et al., 1984; Lehman et al., 2001). Planktonic microbial growth is scarce in this environment. Attachment of the bacteria to solid surfaces results in the development of biofilms. The capability to adsorb to surfaces has been found to be most crucial for survival (Foppen & Schijven, 2006; Signoretto et al., 2004, 2005). A greater decay of bacteria has been demonstrated in the water column than in sediment, suggesting a better survival of attached cells (Craig et al., 2002). The advantage of attached bacteria may be that conditions on surfaces are favourable for nutrient availability due to attached materials and/or a continuous flow of substrates along the surface in addition to the protection function of biofilms (Alfreider et al., 1997; Jin et al., 2000). In the attached state reduced inactivation may result from protection against proteolytic enzymes excreted from other microorganisms or an increased stability of cell envelopes and virus capsids (Gerba, 1984).

Different adhesion rates of bacteria have been detected for different surface materials, the highest activities were observed on synthetic materials (Schwartz et al., 1998). Surface roughness contributed to an increased adhesion (Donlan, 2002; Shellenberger et al., 2002).

Interactions between the hydrophobic parts of microbial and substratum surfaces have been shown to favour adhesion. Greater hydrophobicity of cells and substrata result in greater attractive forces and higher levels of adhesion (van Loosdrecht et al., 1987; Rijnaarts et al., 1993; Schijven & Hassanizadeh, 2000).

One of the physical factors affecting survival is **temperature.** Low temperature was found to be a major factor for prolonged survival of faecal indicator bacteria (Astroem et al., 2007) and *Campylobacter* spp. (Buswell et al., 1998). Survival of viruses and bacteriophages is enhanced below 15° C (Althaus et al., 1982; Blanc & Nasser, 1996; Hurst et al. 1980; Yahya et al., 1993).

Moisture content. Bacterial survival in various types of soil was found to be best during rainy seasons. In sandy soils, which do not retain water for a long time, the survival was shorter (4-7 days) whereas in loam with a higher water retention capacity the bacteria survived for more than 42 days (Gerba & Bitton, 1984). It was shown that water saturation increases the survival and transport of viruses through the subsurface (Collins et al., 2006; Jin et al., 2000). Removal of viruses occurred more extensively during unsaturated conditions. It is attributed to inactivation rather than adsorption (Powelson & Gerba, 1994). The tension at the air-water-interface is suggested to promote virus inactivation.

Effect of pH. Microorganisms survive longer at near neutral pH; a shorter survival has been reported at pH 3-5 (Gerba & Bitton, 1984).

The pH of soil and groundwater was also shown to influence adsorption of microorganisms to soil particles. Usually the surface charge of the protein envelopes of bacteria and viruses is negative. However, at pH under the isoelectric point protons are adsorbed which lead to an overall positive charge and support adsorption to the negatively charged soil particles. Low pH was reported to promote the sorption of viruses to surfaces, a factor beneficial for prolonged survival (Collins et al., 2006; Kinoshita et al., 1993; Schijven & Hassanizadeh, 2000). At decreasing pH the adsorption of Coxsackie, Echo, and Polioviruses increases more than one order of magnitude whereas at pH 8-9 no adsorption is detected (Matthess et al., 1985). Sharp pH increases are responsible for detachment of previously attached viruses (Bales et al., 1991).

Organic matter. High dissolved organic carbon concentration (DOC) has been identified to increase survival rates for bacteria in groundwater microcosms (Cook et al., 2007). Not only DOC itself seemed to be important for the survival of coliform bacteria but also the biodegradability of the compounds in the fluid. This was shown in an assay of different source waters containing equivalent DOC concentrations (Boualam et al., 2003). High organic carbon concentrations as well as high phosphate concentrations have been shown to support a prolonged persistence of *E. coli* (Craig et al., 2002; Juhna et al., 2007). Increasing nutrients (0.1-0.5g/l glucose) or phosphate concentrations enhanced the rate and extent of biofilm accumulation, but still higher concentrations led to a reduction due to detachment (Rochex & Lebeault, 2007).

Under unsaturated conditions, increased survival of virus in the presence of organic matter has been detected by Powelson et al. (1991). They attribute the increased survival rate to the lower surface tension in the presence of organic substances; since there is evidence that air-water surface tension may be an important factor contributing to virus inactivation (Trouwborst et al., 1974).

Not only are the survival rates of microorganisms affected by DOC but also their adsorption behaviour. Viral attachment was found to be reduced in the presence of organic matter because of competition to sorption sites also leading to increased viral movement through the subsurface (Powelson et al., 1991; Zhuang & Jin, 2003).

Dissolved or suspended organic matter consists of proteins, polypeptides, and amino acids as well as humic substances, which can compete with the negatively charged viruses for adsorption sites on the surfaces (Gerba, 1984). Organic compounds in sewage decreased virus adsorption to soil (Lance & Gerba, 1984; Pieper et al., 1997).

Enhanced detachment rates of viruses have been achieved with high concentrations of dissolved organic matter, which is suggested to result from the disruption of hydrophobic bonds between virus and soil. On the other hand, adsorbed organic material and solid organic matter present in the soil may also contain hydrophobic groups on their surfaces, which may provide more binding sites for virus adsorption. Schijven et al. (2002) detected a nonlinear removal of bacteriophages during their passage through soil. The high adsorption within the first meters was attributed to soil organic content. It is suggested that in particular the ferric oxyhydroxides present only at this depth are used as favourable adsorption sites. The positive charge of the solid surfaces allowed adsorption of the negatively charged viruses. Altogether, it seems that the observed effects were variable and not depending on single parameters but rather on a combination of type and concentration of organic matter, of virus type, and of soil type.

Soil microflora. The decay rates of viruses (polio-, coxsackie-, and adenovirus) are enhanced in the presence of indigenous soil and groundwater microorganisms. This may be due to the activity of predators, to differential production of antibiotics and antiviral substances or enzymes (stimulated by the presence of specific nutrients), and/or to antagonistic metabolites amplifying degradation processes (Gordon & Toze, 2003; Sobsey et al., 1980; Wall et al., 2007; Yahya et al., 1993; Yates et al., 1990).

Ionic strength and multivalent cations. Higher ionic concentrations increase the sorption of viruses to soil particles (Moore et al., 1981; Sobsey, 1980). Bacterial adhesion rates increased with increasing ionic strength of the medium, too (Won et al., 2007). In the presence of high concentrations of multivalent cations attachment is increased specifically in highly negatively charged virus types (Dowd et al., 1998). Virus attachment to quartz was shown to increase when multivalent cations (Ca or Mg) were present in the water instead of a monovalent Na-ion.

Dissolved oxygen. Maximum decay of *E. coli* and various viruses under aerobic conditions was detected by Gordon & Toze (2003). They suggested that the presence of oxygen and nutrient levels indirectly influence microbial decay by influencing the activity of the groundwater microorganisms

Under anoxic conditions inactivation rate of bacteriophages was found to be much lower than under oxic conditions (van der Wielen et al., 2006)

Major factors for the survival and persistence of microorganisms in the subsurface are summarized in Table 9:

Table 9: Factors affecting survival of enteric bacteria and viruses in soils and groundwater

Factor	**Enteric bacteria**	**Virus/Bacteriophages**
Adsorption to mineral surfaces	Increased survival by increased attachment, die-off is reduced	Survival time is enhanced when viruses are adsorbed to soil
Temperature	Longer survival at low temperatures, longer survival in winter than in summer	One of the most detrimental factors, enhanced survival at temperatures below 15° C in GW
Moisture content	Desiccation is detrimental to most microorganisms (spores and cysts excepted), longer survival in moist soils and during times of high rainfall	Increased virus reduction in drying soils (inactivation at the air-water interface)
pH	Longer survival at near neutral pH, shorter survival time in acid soils (pH 3-5) than in alkaline soils, low pH favours adsorption and thus survival	Survival may be prolonged at near neutral pH, indirect survival effect: low pH and high ionic concentrations increase adsorption to soil particles
Organic matter	Increased survival by adsorption and possible regrowth when sufficient amounts of organic matter are present	In unsaturated zones: organic matter reduces tension at the air-water interface and thus reduces virus inactivation
Soil microflora	Increased survival in sterile soil, lower survival in natural environment, due to protozoa/antagonists, competition for nutrients, or inhibitory or damaging substances secreted by other microorganisms	No clear trend in soil, microbial activity diminishes the survival of viruses in groundwater
Cations	Increase in cationic concentration favours adsorption (capacity for binding and the strength of bonds are affected)	Certain cations have a thermal stabilizing effect on viruses and increase survival. Indirect influence by increased adsorption to soil, survival of virus better in adsorbed state
Dissolved oxygen level (DO)	Low DO levels might impede predatory eukaryotes or antagonistic bacteria, survival of enteric bacteria would be enhanced (they are microaerophilic and low DO content should not be detrimental)	no information

Adapted from Gerba & Bitton (1984); WHO report (2003); Collins et al. (2006); John & Rose (2005); Pedley et al. (2006) and references in the text.

Microbial variables. Bacterial surface characteristics like flagellated cells provide better attachment properties and lead to a higher rate of colonization as compared to unflagellated cells (Mueller, 1996). On the other hand cells under starvation conditions have a reduced size resulting in reduced adhesion and increased motility.

The size and shape of bacteria influence the rate of transport. Larger cells are thought to be removed more efficiently by filtration. The hydrophobicity and electrostatic charge of the cell walls interact with particle surfaces (Lawrence et al., 1996) and determine their adsorption capabilities. Temporal changes in nutrient supply and other environmental conditions, often encountered in the subsurface, might induce different metabolic activities in the bacteria as a contribution to survival. Such changes might lead to modified surface structures with the production of extracellular polymeric substances (including flagella and pili) and different LPS composition (lipopolysaccharides) in the outer membrane, which increase adsorption and biofilm formation. The production of spores is connected to a higher potential for surviving dry periods. Starvation times might induce cell size reduction, fragmentation (increase in cell numbers due to division without growth), higher cellular motility resulting in higher diffusivity and faster rate of movement (*Pseudomonas* species analysed by Mueller, 1996; *E. coli* by Arana et al., 2004), and increases in hydrophobicity and irreversible binding capacity (Kjelleberg & Hermansson, 1984).

The ability to change the metabolism under adverse environmental conditions to the VBNC state (viable but not cultivable) represents the ultimate strategy for survival (*Enterococcus*: Lleo et al., 2005a, b, 2003; *Campylobacter jejuni*: Cook et al. 2007). In this state microorganisms cannot be cultivated but nevertheless maintain some metabolic capacities for some time, e.g. the ability to synthesize polymeric substances (Lleo et al., 2007). Upon return to favourable conditions, viability and pathogenicity are restored.

In Table 10 a comparison is shown between the elimination rates of some bacteria and that of *E. coli*. Great differences in die-off rates have been found. In the groundwater environment bacteria like *Clostridium perfringens* and *Streptococcus faecalis* seem to survive longer than *E. coli*, whereas *Staphylococcus aureus* and *Salmonella typhimurium* are eliminated more rapidly.

Table 10: Relative die-off of pathogens compared to E. coli in groundwater (10± 1°C) at a reduction of seven orders of magnitude.

Microorganisms	Time (days)[1]	Elimination constant λ_t (d^{-1})	Relative elimination compared to *E. coli* $\lambda_t / \lambda_{t\ E.\ coli}$
Escherichia coli	225	0.0522	1
Salmonella typhimurium	290	0.0564	1.1
Yersinia enterocolitica	300	0.0167	0.3
Pseudomonas aeruginosa	295	0.0204	0.4
Staphylococcus aureus	20	0.484	9.3
Streptococcus faecalis	300	0.0291	0.6
Bacillus cereus	300	0.0218	0.4
Bacillus megatherium	5	2.61	50
Clostridium perfringens	300	0.00491	0.09

Adapted from Filip et al. (1985)

⇨ In summary, due to the metabolic state and their individual capacity to cope with adverse environmental conditions, the different microorganisms have a different survival probability.

Chapter 5 Parameters from well design and well operation that increase the risk of microbial contamination

In order to assess the susceptibility of the well to microbial contamination, spatial and temporal aspects of all flows including inputs and outputs from groundwater and associated wells must be considered in addition to the geological setting.

Control of waterborne microbial pathogens starts by protecting the source water to prevent faecal contaminants from entering aquifers and wells. The installation of protection areas around the wells serves this purpose. In Germany, protection zone I extends 10 m round the well. It serves the protection of the immediate surrounding of drinking water extraction wells from contamination. Here, only measures to secure the water production are allowed. Protection zone II extends to the line from where the groundwater requires 50 days to reach the well. In particular, building activities, the deposit of waste and sewage, and permanent grazing of animals are inhibited. Protection zone III extends to the limits of the subsurface discharge area, representing the complete aquifer (DVGW Arbeitsblatt W101). No activities are allowed which may be detrimental to water quality.

Planning, construction, and operation of wells are subject to a number of regulations. In complying with these rules and considering the best management practices during operation and monitoring of the project, contamination events should be avoided or kept at a minimum.

5.1 Risk from well design

Influence of well construction. Wells are dug, bored, or drilled. During each procedure, microorganisms can be introduced into the aquifer and the contamination from operating tools could lead to microbial transfer from one site to the next if proper disinfection is not executed.

Dug and bored wells are more vulnerable to contamination in locations of shallow soil profiles and high water tables due to the texture of the borehole (Conboy & Goss, 2000).

A negative correlation of coliform bacteria levels to the casing length was found for some investigated wells (Tuthill et al., 1998).

Wells constructed with open-jointed casings (concrete, brick, or tile) were shown to be more susceptible to contamination whereas PVC and steel cased wells in contrast showed lower susceptibility (Gosselin et al., 1997). They also stated that improperly constructed wells represented a major contribution to contamination of domestic well water. In such cases, enhanced vulnerability of wells may be expected when gravel packs do not provide adequate pore sizes for the environment, allowing the passage of contaminants from the surface. In addition, improper casing diameters may lead to disturbances due to water discharge being too high for the site.

During construction care should be taken that the equipment and materials for the well (e.g. pipes, joints, gravel packs) are not left in the open but are wrapped and protected from contamination from the surface until use.

Influence of materials. Wells should be constructed with materials that have the least potential to be colonized by microorganisms in order to avoid or minimize the formation of microbial biofilms and microbial induced clogging and corrosion.

Schwartz et al. (1998) analysed the biofilm formation on different materials and found that copper and steel were less colonized by microorganisms than PVC and polyethylene. In general, rough surface texture tends to be preferentially colonized.

Well depth. This seems to have a strong impact on susceptibility to contamination. Gosselin et al. (1997) reported that in deeper situated wells water quality usually was better, due to the longer migration pathways of contaminants from the surface. Verstraeten et al. (2005) reported predominant contamination of shallow domestic sand-point wells. Impacts of bacteria on well water were generally more prominent in shallow wells and beneath a shallow soil profile whereas in deep wells typically contamination was detected less often (Conboy & Goss, 2000; Francy et al., 2000; Oliphant et al., 2002; Paul et al., 1995; Wireman & Job, 1998).

Well age. It has been shown in a comparison of wells that older wells (e.g. 50 years old, reported by Gosselin et al. 1997) possess a statistically higher probability to receive contamination than those more recently built (Conboy & Goss, 2000).

- ⇨ During well construction, hygiene needs to be ensured.
- ⇨ Old wells, wells made of PVC or PE, wells built with open casings (bricks, concrete or tiles) and shallow wells have shown enhanced susceptibility towards microbial contamination.

5.2 Risk from well operation

Discharge management. For each well an appropriate discharge system is proposed which takes into account the specific characteristics of the well and the aquifer. Different discharge quantities and duration of discharge may have an impact on the quality of well water. It has been shown that in prolonged times of no discharge biofouling processes may start. Enforced discharge of the well may lead to an increased nutrient supply (for microorganisms attached to the surfaces around the well) and in this way support bacterial growth. On the other hand, patches of mature biofilms may detach under high flow velocities and pollute the well water. Alternating discharge and no discharge may induce turbulences thereby increasing oxygen concentration, which may provide favourable conditions for the proliferation of iron-oxidizing bacteria and promote clogging.

Pumping of the well can reverse natural flow directions of the groundwater system, with the effect that the influx of a potential contamination may derive from a different direction.

Maintenance. Procedures like servicing and repair of the well facilities always represent an increased risk of contamination because various manipulations have to be performed with open casings and open wellheads. Gelinas et al. (1996) reported in a study of well water in Guinea that insufficient well maintenance was the main factor contributing to the microbiological contamination. Wellhead vulnerability and management practices were considered possible sources of contamination (Conboy & Goss, 1999; Macler & Merkle, 2000).

Monitoring. Control of the well water quality may involve risks resulting from sampling procedures not always corresponding to the best management practices (e.g. sterile handling). A history of the contamination events has to be kept and a defined sampling schedule established in order to trace the microbial pollution. For a permanent contamination control, the constructive monitoring is also important. This term includes the determination of the current state of the well pipes, pipe connections, sealing, gravel filled annular space, the well shaft, as well as possibly existing hydraulic short cuts. After conclusion of these procedures, the result of a microbiological examination of the well water has to be awaited before the well is allowed to be put in operation.

- ⇨ Highly fluctuating well discharges are favourable towards microbial contamination,
- ⇨ During well maintenance hygiene needs to be ensured,
- ⇨ Well monitoring with respect to microbial contamination includes careful documentation, sampling and constructive monitoring.

Chapter 6 Techniques and analytical methods that can be used to determine the sources and pathways

Investigations of intrinsic or polluting microorganisms in groundwater and wells, in planktonic form or attached to surfaces as biofilms, essentially rely on two types of methods: cultivation techniques and molecular methods. Culture-based methods usually account for only a minor part of the resident microbial community in all sites. This is specifically true under oligotrophic conditions where microorganisms are difficult if not impossible to culture. Reasons are ill-defined nutrient demands and chemical properties of the water, or possible requirements to grow in specific biofilm microenvironments (Ross et al., 2001) which cannot easily be provided in the laboratory. The frequently encountered dormant or VBNC state of the bacteria also hinder an effective cultivation of the microbial inhabitants of groundwater and well (Rollins & Colwell, 1986). The difficulty to detect a representative number of microorganisms in a given microbial habitat by cultivation has been supplemented by the use of molecular methods (Chatzinokas & Harms, 2003; Hahn, 2006). Here the physical presence of specific microbial sequences are investigated, however they provide no information on their viability. The methods in addition imply biases concerning the efficiency and selectivity of DNA extraction, differential sequence amplification, and operon heterogeneity (Wintzingerode et al., 1997). Microbial diversity analysed by cultivation techniques might present a different population composition than that derived from a cultivation independent approach. Thus, depending on the requested aim, the most appropriate methods should be chosen.

6.1 Cultivation-based methods

Detection of indicator bacteria generally relies on cultivation-based methods, such as:

Colony counts. Quantitative detection of cultivable microorganisms can be achieved by enumeration on nutrient agar followed by incubation for 44 hours at 36° C and parallel for 68 hours at 22° C under aerobic conditions (DIN EN ISO 6222) resulting in the growth of colony forming units (cfu). Different culture media result in the detection of different groups of bacteria. This method is applied for the control of drinking water.

MPN (most probable number) technique. This method is based on the incubation of different dilutions of bacterial samples in a defined medium containing indicator compounds and subsequent detection of the metabolic activity of the bacteria. It is applied for the detection and enumeration of *E. coli* and coliform bacteria in surface and wastewater (DIN EN ISO 9308-3). The sample is incubated in a liquid medium at 44°C for 36 to 72 hours on microtiter plates. For the detection of *E. coli,* the medium contains MUG (4-methylumbelliferyl-ß-D-glucuronid) which is hydrolyzed by the *E. coli* enzyme ß-D-glucuronidase and the product is visualized as a fluorescent compound under UV light.

For the detection and enumeration of intestinal enterococci, the medium contains MUD (4-methylumbelliferyl-ß-D-glucosid), which is hydrolyzed in the presence of thalliumacetate, nalidixic acid, and TTC (2,3,5-triphenyl-2H-tetrazoliumchloride) to a fluorescent compound also detectable under UV-light (DIN EN ISO 7899-1).

Membrane filtration. This method is based on the filtration of a defined volume of water sample through a membrane filter (pore size 0.45µm) which is subsequently placed on agar plates with selective medium supplemented with indicator substances. It is described for the detection and isolation of the intestinal enterococci species *E. faecalis*, *E. faecium*, *E. durans*, and *E. hirae* (DIN-EN-ISO 7899-2). Here the medium contains sodium azide (to prevent growth of Gram-negative bacteria) and TTC (colourless) that can be reduced to formazan (red colour) by enterococci. Other enterococci and streptococci not originating from faecal pollution might also be cultivated.

In order to confirm specifically the presence of intestinal enterococci, the filter with the colonies is transferred to bile-aesculin-azide containing agar plates. When these bacteria are present, they hydrolyze aesculin at 44° C within two hours producing 6, 7-dihydroxycumarin, which is complexed with ferric iron, and are then detectable as a coloured compound in the medium.

The Chromocult Enterococcus Agar has been classified as equivalent by the German Federal Environment Agency and represents an accepted method. The advantage is reduced work and timesaving (Lange et al., 2007).

Membrane filtration for the detection and enumeration of *E. coli* and coliform bacteria is described in DIN EN ISO 9308-1**.** The test consists of two parts. The basic part consists of the filtration of the water sample followed by cultivation at 36° C for 24h on selective lactose-TTC-agar medium (lactose positive bacteria show yellow colour in the medium) which is then supplemented by biochemical characterization of the resulting typical colonies. For that purpose, the colonies are incubated at 36° C for 24h on non-selective agar for the oxidase test and at 44° C on tryptophane-containing medium for the formation of indole (red colour). Oxidase-negatives represent coliform bacteria; those with additional positive indole test are taken as *E. coli*. As the selectivity of the medium is low, this method is preferably used for samples with low bacterial content. A high accompanying background flora might render the evaluation of the results impossible.

In a fast test, the filter membrane is first incubated on TSA-medium (trypton-soy-agar) at 36° C for 4-5h and then transferred to TBA (tryptone-bile-agar) at 44° C for 19-20h. The membrane must subsequently be tested for indole production to determine the number of *E. coli* present.

The detection of *Clostridium perfringens* (including spores) is performed by membrane filtration, followed by incubation on specific agar at 44° C for 24h under anaerobic conditions. The dark yellow colonies are then exposed to ammonium hydroxide and should change to red colour within 20-30 seconds.

Colilert method. Colilert 18/Quanti-Tray is a commercially available product (Firma Idexx Laboratories, USA) for the simultaneous detection and enumeration of coliform bacteria and *E. coli* directly from water samples (a yellow colour denotes the presence of coliforms, fluorescence at 366 nm indicates the presence of *E.coli*). It is based on the MPN technique and includes the substrates MUG as well as o-nitrophenyl-ß-D-galactopyranoside (ONPG), which is hydrolized by the enzyme ß-galactosidase and produces a yellow chromogen.

The method is used as an alternative to ISO 9308-1 in Germany and represents a validated method in the US (Edberg et al., 1990; Edberg et al., 1991; Olson et al., 1991; Bonadonna et al., 2007).

BART-ests. These **B**iological **A**ctivity **R**eaction **T**ests (Cullimore, 2000) represent a number of test kits for the detection of different groups of bacteria, e.g. responsible for well clogging processes in water. The methods determine the metabolic activities of the bacteria over time and thus allow an approximate assessment of microbial abundance present in the sample.

Test kits are available for bacteria often recognized in subsurface habitats such as heterotrophic aerobic bacteria, iron-reducing bacteria (Bartetzko, 2002), sulphate-reducing bacteria, nitrifying and denitrifying bacteria. They give an idea what kind of clogging or corrosion can be expected in a given well system.

Bacteriophage detection. The detection and enumeration of phages in environmental samples consists in a concentration step of the sample, followed by the detection assay, which is performed by infecting a culture of the bacterial host plated on agar, and subsequent incubation at appropriate temperature.

The resulting plaques are counted as plaque forming units (pfu) for F-specific RNA-bacteriophages and host strain *Salmonella typhimurium* WG49 (DIN EN ISO 10705-1), and for somatic bacteriophages and host strain *E. coli* C or WG5 (DIN EN ISO 10705-2).

Detection of enteric and pathogenic viruses. The cell culture technique has been the most widely used method for the detection and quantification of infectious viruses. A cell line adequate for the propagation of a given virus is chosen for inoculation with the sample, producing a cytopathic effect (morphological alterations or destruction of the cells) if viable virus particles are present.

6.2 Molecular methods

In the last years, molecular methods have been developed which target the detection of specific sequences in the bacterial or viral genome without the need for cultivation. An advantage is the rapid and sensitive detection of the microbial presence however without an indication of viability of infectivity.

PCR (polymerase chain reaction), RT-PCR (reverse transcription PCR). The method is based on the amplification and detection of specific sequences in bacterial or viral DNA/RNA with broad homology within a specific group. A variety of primers (short complementary sequences) have been developed for the detection of many different bacteria and viruses.

Microorganisms can be detected by PCR targeting a variety of genes such as lacZ gene (diagnostic for coliforms), uidA gene (coding for ß-glucuronidase, diagnostic for *E. coli* and *Shigella*), lamB genes (detection of *E. coli*, *Salmonella*, *Shigella*) (Bej et al., 1990, 1991a), hemolysin gene, Shiga toxin from *E. coli* (Welinder-Olsson et al., 2005), specific coliphages (Kirs & Smith, 2007), human and bovine enteric viruses (Abbaszadegan et al., 1999; Fong et al., 2005), or for the 16S rRNA genes (Schmalenberger et al., 2001; Weisburg et al., 1991).

The identities of the products are either confirmed by hybridization to specific probes or a nested PCR is followed which uses a second round of amplification to increase the PCR product to levels detectable by electrophoresis. Multiplex PCR involves simultaneous amplification of different gene sequences (Bej et al., 1991b).

Real-time PCR provides quantitative information about the targeted sequences (Frahm & Obst, 2003; Fuhrman et al., 2005). A specific application is the ICC-PCR (integrated cell culture) which is used for the detection of virus in cell cultures that do not show direct cytopathic effects upon infection with the virus.

DGGE (denaturing gradient gel electrophoresis). This analysis performs the separation of the 16S rDNA fragments produced by PCR and allows the comparison of the microbial diversity in different habitats due to different electrophoretic patterns (Kuhlmann et al., 2000).

FISH (fluorescent in situ hybridization). This method is used for the *in situ* detection and enumeration of specific groups of bacteria. The method is based on the chemical coupling of general or group specific oligonucleotide probes to fluorescent dyes (e.g. cy3) followed by hybridization to complementary sequences in the cells (Bade et al., 2000; Behrens et al., 2003). Detection is performed by epifluorescence microscopy or flow cytometry. FISH targets rRNA and is thus affected by cell wall properties, cellular ribosome content, the number of rRNA operon copies per cell, and the accessibility of rRNA during hybridization.

Determination of cell counts. Total cell numbers in the samples can be detected by direct epifluorescence microscopy after staining the bacteria with DAPI (4,6-diamino-2-phenylindol), acridine orange, or CTC (5-cyano-2,3-ditolyl-tetrazoliumchlorid).

6.3 Microbial source tracking

The methods described below have been used for tracking the type of health related microbial contamination by targeting either the pathogens or the indicator microorganisms instead. As the occurrence of indicator microorganisms is not restricted to human sources but extends also to other warm blooded animals, either domestic animals or wildlife, it might be important to discriminate between the origins of the diverse contaminations.

The knowledge of defined pollution sources might aid in the prevention of contamination and represent a first step to restore water quality. These types of analyses have been collectively named microbial source tracking (Cimenti et al., 2007; Taylor & Ebdon, 2007; Plummer & Long, 2007). The success of this approach is however dependent on several assumptions (Bitton, 2005; Gordon, 2001; Harwood, 2007):

- members of a given species have adapted to living in a specific host or under specific environmental conditions
- the clonal composition of the population differs with the habitat
- microbial strains display host specificity, a particular clone is more likely to be isolated from one host species than from another
- the clonal compositions of populations are stable and can be recovered for extended periods of time

Based on these postulates, genotypic and phenotypic differences in microbial strains, resulting from their existence in different hosts or habitats, are shown in a fingerprint that can in some cases be attributed to a particular host group or environment. Two different types of methods are applied: one type is cultivation-based and depends on extensive database of habitat-specific characteristics of the populations, whereas the other depend on library-independent methods which concentrate on host-specific characteristics (Blanch et al., 2006; Field & Samadpour, 2007; Hamilton et al., 2006; Myoda et al., 2003; Stoeckel & Harwood, 2007).

The determination of multiple **antibiotic resistance patterns** (MAR) of coliforms has been used for the discrimination between human and nonhuman sources of faecal coliforms and streptococci (Guan et al., 2002; Harwood et al., 2000; Wiggins, 1996; 1999). Hagedorn et al. (1999) found correct classification rates of >95% for the separations between animal and human sources with also a potential to discriminate between different types of animals depending on the database for the given environment.

Carbon utilization profiles of enterococci have been used to identify the different metabolic capabilities of human versus non-human origins by using microplate systems (e.g. BIOLOG) with substrates for microbial growth (Hagedorn et al., 2003).

A combination of **serotyping** and PFGE (pulsed-field gel electrophoresis) has been used to discriminate Campylobacter isolates from patients, drinking water and other environmental sources by (Clark et al., 2003; Haenninen et al., 2003).

A combination of PCR and **RFLP** (restriction fragment length polymorphism) has been used to discriminate between human/ruminant and human/cow sources of faecal contamination (Bernhard & Field, 2000a, and b; Bower et al., 2005).

Conboy & Goss (2001) found *Clostridium perfringens* as a reliable indicator of contamination from animal manure. A number of methods, including **ribotyping** (Carson et al., 2001) and **variations of PCR** protocols have been applied for microbial source tracking. In Table 11 some of these approaches have been assessed for their reliability to correctly identify the different sources of the targeted *E. coli*.

Table 11: E. coli isolates correctly identified to source by use of genetic and phenotyping methods

Bacterial source	Genetic fingerprint (in %)[1]						Pheno-typic
	Ribo-typing	AFLP	16S RNA	BOX-PCR	Rep-PCR	ERIC-PCR	MAR
livestock[2]	78	94-98	78	93	57	37	46
wildlife[3]	--	97	74	--	--	--	95
human	93	91-97	80	95	90	29	55

[1]Adapted from Tallon et al. (2005), and other references: Carson et al. (2001); Guan et al. (2002); Leung et al. (2004); Dombek et al., (2000).

AFLP, amplified fragment length polymorphism; BOX-PCR, highly conserved repetitive DNA sequences PCR with BOX primers; Rep-PCR, repetitive DNA element PCR with repetitive extragenic palindromic primers; ERIC, enterobacterial repetitive intergenic consensus.

[2]includes averages for combinations of pigs, geese, cattle, chicken or turkeys.

[3]includes the combined result averages for moose and deer.

In a multilaboratory study 26 parameters were analysed including the enumeration of faecal coliform bacteria, enterococci, clostridia, somatic coliphages, F-specific RNA phages, bacteriophages infecting *Bacteroides fragilis*, *B. thetaiotaomicron*, and bifidobacteria; genotyping of F-specific RNA phages; and measurements of faecal sterols (Blanch et al., 2006). As a result it was concluded that single parameters were not enough to discriminate the source of faecal pollution from humans and animals, instead a combined analyses of a series of parameters are required for reliable results.

- ⇨ There are molecular methods available, that distinguish between micro-organisms resulting from human and from animal faeces.
- ⇨ However, only a combined analyses of a series of parameters yields reliable results.

6.4 Other methods

Well water parameters have been analysed in order to examine whether a correlation to microbial contamination could be established. A combination of microbiological and nitrate values have been found useful as an indication of sewage contamination (Lerner et al., 1990; Barrett et al., 1999). Oliphant et al. (2002) observed significant correlations of the concentration of chloride, coliforms and nitrates to faecal coliform contamination through septic systems effluents.

- ⇨ Sewage indicators like nitrate or chloride might be useful to identify a microbial contamination through sewage.

Methods for the detection of recharge water

Analysis of the contribution of surface water to well water was performed by determination of the stable isotope ratios of the water (Hunt et al., 2005). Travel time of surface water to the well was analysed by measurements of temperature, virus culture, particle tracking. This information is used for an assessment of the vulnerability of wells to contamination.

- ⇨ If surface water is the source for microbial contamination of a well, travel times obtained from tracer measurements can be used to assess the vulnerability.

Chapter 7 Summary and Conclusions

This report has presented a view on microbial life in the subsurface, including indigenous and polluting microorganisms, together with their possible origins, their migration paths as well as the problems they may pose for the quality of abstracted water from the wells and their impact on human health.

- Microbial occurrence and the composition of microbial communities in groundwater are variable depending on the heterogeneous properties of the soil and the aquifer.
- Major determinants for microbial abundance and activity in groundwater are nutrition supply, availability of dissolved oxygen and energy sources. Environmental factors like temperature, pH, hydrostatic pressure, dissolved salts influence microbial persistence.
- The same processes and factors that influence the quality of groundwater should also be relevant for well water. Wells represent in addition a specific habitat for microorganisms with different attachment surfaces, increased water flow, and availability of space.
- Under the oligotrophic conditions often present in groundwater and wells, most microorganisms are attached to surfaces.
- It is not feasible to track faecal contamination by determining the multitude of different pathogens with variable characteristics. Instead, bacteria are tracked, which generally are most abundant in faeces (like *E. coli*), thus indicating faecal pollution. This indicator principle has been universally accepted. It is agreed upon that the occurrence of faecal indicator bacteria does not always coincide with the presence or absence of pathogenic bacteria and even more so for pathogenic viruses and protozoa. Usually the abundance of indicators is taken as a measure of probability for the presence of pathogens. Debate remains about the use of bacteriophages as indicators for the presence of pathogenic viruses. The correlation of indicator to pathogen should be assessed for each specific environment because of their different transport and survival characteristics.
- The introduction of microorganisms into the subsurface occurs with recharge water, typically from precipitation or infiltration of surface waters. Major sources of microbial pollution in groundwater were reported to result from leakages of sewer facilities, from animal wastes, from faecal contamination of surface soil and surface water (human, animal, wildlife). Additional contamination, specific for wells, may arise from unhygienic manipulations during operation and/or monitoring procedures.
- Depending on the subsurface material, such as pore sizes, presence of canals, hydrophobicity of surfaces, migration velocity can be very different. Once below the water table, the vertical movement diminishes. Further migration is determined by the direction and velocity of the aquifer flow. During the passage, the distance of microbial migration is dependent on various processes such as filtration, dispersion, attachment to surfaces, and die-off.
- Important for the assessment of pathways, with respect to the speed and distance of microbial subsurface passage, is a detailed knowledge of the structural conditions of the aquifer and the survival times of the microorganisms involved.
- Various biochemical, microbiological, and molecular methods have been developed and are ready to be used for the determination of the types, abundance, and metabolic activities in microbial communities, for the tracing of

faecal pollution with indicator microorganisms, and for the detection of specific pollution sources.

- Sensitivity of wells to contamination is principally a function of the hydrogeological and geochemical site characteristics of the specific location of the well, the overlaying saturated and unsaturated subsurface zones, the distance to potential contamination sources and the correct functioning of wellhead protection, ascertained by best management practices during operation and monitoring.

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